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Impact of food processing and gut transit on *Enterococcus faecalis* virulence gene expression

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Summary

Enterococcus faecalis is a multifaceted organism capable of inhabiting a diverse spectrum of environmental niches. Due to its beneficial application in foods and as a probiotic, healthy human hosts are in frequent contact with a variety of *E. faecalis* strains. Yet, this organism is also commonly associated with a number of different nosocomial infections. While a direct link has not yet been established between the consumption of *E. faecalis* and the development of foodborne infection, the ability of this bacterium to cause disease in humans and the frequency with which it both harbors and passes on antibiotic resistance genes makes the use of *E. faecalis* in food applications a concern. As yet, the mechanisms involved in this organism's conversion from benign commensal, fermentation organism or probiotic to pathogen are undetermined, and the identification of these mechanisms is complicated by the fact that some traits, which contribute to its pathogenicity, are also useful in food and probiotic applications (i.e. the ability to survive food processing conditions including hyperosmotic, acidic, high temperature and high hydrostatic pressure stresses, in addition to the acidic and bile salts stresses encountered in the host gastrointestinal tract). Thus, decisions regarding the safety of *E. faecalis* for human consumption are difficult to make, particularly in the face of the current push for greater regulation of bacteria used in food applications, and prompted this study of how different environments found both in foods and the host affect *E. faecalis* virulence gene expression.

The approach to this study of *E. faecalis* virulence involved comparison of gene presence and regulation in response to factors active in food environments and gut transit in strains of *E. faecalis*. Isolates from different environments (e. g. food, clinical infections) were screened for the presence of established virulence genes to determine if the origin of isolation affects the number or type of virulence gene(s). A phenotypic analysis was also conducted, which revealed that although bacteria possess virulence genes, they are not always

expressed. Environmental studies included various conditions found in fermented sausages and cheeses, simulated *in vitro* conditions of the host, contact with (cultured) host intestinal epithelial cells of both mouse and human origin, and an *in vivo* mouse model of *E. faecalis* infection. Additionally, since food processing conditions as well as the host intestinal environment can result in a sub-lethally stressed population of cells, the level of virulence gene expression by this defined population of *E. faecalis* was determined in response to these environments (high salt, acid pH, heat, nitrite, HHP, bile salts). With this wide spectrum of environmental conditions a comprehensive picture was obtained of how *E. faecalis* regulates established virulence genes during its trip from food to the site of infection.

Although the results of these studies do not provide a simple answer to the question of how a pathogenic *E. faecalis* can be distinguished from a "safe" *E. faecalis* strain, one can make several conclusions based upon these results. First, (1) the origin of isolation does not correlate with the number or type of virulence gene(s), (2) all isolates are originally of fecal origin and there are no specific "food" or "clinical" isolates. Additionally, (3) the presence of virulence genes does not necessarily render a strain "unsafe" for use in food applications, as they are not always expressed, and (4) the immune state of the host also influences the level of virulence gene expression. Thus, one must keep a perspective on the significance of these negative aspects associated with food bacteria (LAB) to avoid the creation of a *horror vacui*, (freely translating to: fear of underregulation) in which the minute details of a few negative traits obscure the many positive qualities that these organisms, including *E. faecalis*, have long imparted to foods. On the other hand, preventative measures can be taken as a precaution and incorporated into criteria for starter and probiotic culture selection and HACCP guidelines during food production, which include the use of *E. faecalis* strains that lack both *fsrB* (this gene appears to regulate the expression of several virulence genes including *gelE* and *efaAfs*) and *agg* expression (this gene is involved in the spread of both

antibiotic resistance and virulence genes), and the regulation of the intensity and order of applied stresses (i.e. food processing conditions) to avoid pre-conditioning *E. faecalis* for virulence.

Zusammenfassung

Enterococcus faecalis ist ein vielseitiger Organismus, der unterschiedlichste Habitate besiedeln kann. Als natürlicher Bestandteil der Darmflora sowie aufgrund seiner Verwendung als Starterkultur bzw. als Probiotikum, stehen *E. faecalis* Stämme ständig in engem Kontakt mit dem gesunden Menschen. Andererseits sind diese Organismen auch häufig an nosokomialen Infektionen beteiligt. Bisher konnte jedoch noch keine direkte Verbindung zwischen der Aufnahme von *E. faecalis* und dem Ausbruch einer Lebensmittelinfektion hergestellt werden. Dennoch ist die grundsätzliche Fähigkeit dieses Bakteriums, Krankheiten im Menschen zu verursachen und die Häufigkeit, mit der es Antibiotika-Resistenzgene sowohl trägt als auch weitergibt, Anlass zu Bedenken hinsichtlich seiner Anwendung in Lebensmitteln. Bisher sind die Mechanismen, die am Übergang vom gutartigen Commensalen, Fermentationsorganismus oder Probiotikum zum Pathogen beteiligt sind, unbekannt. Die Identifizierung dieser Mechanismen wird zudem dadurch erschwert, dass manche Eigenschaften, die zur Pathogenität beitragen, bei Lebensmittelfermentationen und probiotischen Anwendungen nützlich sind, z.B. die Fähigkeit Lebensmittelverarbeitungsbedingungen, einschließlich hyperosmotischen-, Säure-, Hochtemperatur- und Hochdruckstress sowie Säure- und Gallensalzstress im Gastrointestinaltrakt des Wirtes zu überleben. Deswegen ist die gesundheitliche Unbedenklichkeit von *E. faecalis* für den menschlichen Verzehr, insbesondere angesichts der gegenwärtigen Anstrengungen hin zu einer umfassenderen Regulierung der zu verwendenden Bakterien in Lebensmittelanwendungen, schwer abzuschätzen. Hieraus erwächst die Triebfeder für dieser Arbeit, den Einfluss unterschiedlicher Umweltbedingungen in Lebensmitteln und im Menschen auf die Virulenzgenexpression von *E. faecalis* zu untersuchen.

Diese Arbeit zur Virulenz von *E. faecalis* beinhaltet den Vergleich der Genexpression und –regulation als Antwort auf Einflussgrößen die in der Lebensmitteln und deren Verarbeitung, sowie bei der Darmpassage auftreten. Isolate unterschiedlicher Herkunft (z.B. Isolate aus Lebensmitteln und aus dem Zusammenhang mit klinischen Infektionen) wurden hinsichtlich des Vorhandenseins bekannter Virulenzgene untersucht, um zu bestimmen, inwieweit eine Korrelation zwischen der Herkunft des Isolates und der Anzahl bzw. Art der Virulenzgene besteht. Eine phänotypische Untersuchung zeigte, dass nicht alle vorhandenen Virulenzgene auch immer phänotypisch exprimiert werden. Untersucht wurden sowohl Umwelteinflusse, wie sie in fermentierten Würsten und Käse vorkommen, als auch der Einfluß *in vitro* simulierter Bedingungen des Wirtsorganismus, Bedingungen des Kontakts mit (wachsenden) intestinalen Epithelzellen aus Maus und Mensch und eines *in vivo* Mausmodels einer *E. faecalis* Infektion. Da sowohl Bedingungen wie sie in Lebensmitteln bzw. deren Herstellung auftreten als auch das intestinale Milieu des Wirtsorganismus zu einer Population von subletal gestressten Zellen führen können, wurde zusätzlich die Virulenzgenexpression in dieser Population von *E. faecalis* in Abhängigkeit von hoher Salzkonzentration, niedrigem pH-Wert, Hitze, Nitrit, hohem hydrostatischen Druck und Gallensalzen bestimmt. Mit diesem breiten Spektrum an Untersuchungsbedingungen, konnte ein umfassendes Bild der Regulation von bekannten *E. faecalis* Virulenzgenen während der Passage vom Lebensmittel hin zum Infektionsort erhalten werden.

Obwohl die Ergebnisse keine einfache Antwort auf die Frage wie pathogene *E. faecalis* von „sicheren“ *E. faecalis* Stämmen unterschieden werden liefern können, so lassen sich doch aus den erarbeiteten Daten verschiedene Schlussfolgerungen ableiten. (1) Die Herkunft des Isolats korreliert nicht mit der Anzahl oder dem Typ der Virulenzgene. (2) Da alle Isolate ursprünglich fäkalen Ursprungs sind gibt es keine spezifischen „Lebensmittel“- oder „klinischen“ Isolate. (3) Allein das Vorhandensein von Virulenzgenen macht einen

Stamm nicht notwendigerweise für den Einsatz in der Lebensmittelproduktion “unsicher”, da diese nicht immer exprimiert werden, und (4) der Immunstatus des Wirts beeinflusst ebenso das Ausmaß der Virulenzgenexpression. Der Stellenwert dieser negativen Aspekte, die mit Fermentationsorganismen wie *E. faecalis* verbunden sind ist vor diesem Hintergrund differenziert zu bewerten. Hierbei ist einerseits die Entstehung eines *horror vacui* (frei übersetzt: Angst vor Unterregulierung) zu vermeiden, in dem die geringsten Hinweise auf wenige negative Eigenschaften die vielen positiven Qualitäten für den Menschen angewendter Organismen, eingeschlossen *E. faecalis*, das letztendliche Maß für deren Unbedenklichkeit ergeben. Andererseits können vorbeugende Maßnahmen getroffen werden, indem Selektionskriterien für Starter- und probiotische Kulturen und die Erstellung von HACCP Konzepten für die Lebensmittelproduktion erarbeitet werden. Erstere sollten beinhalten, dass nur *E. faecalis* Stämme Anwendungen finden, die keine *fsrB* (dieses Gen scheint die Expression von verschiedenen Virulenzgenen, einschließlich *gelE* und *efaAfs*, zu regulieren) und *agg* Expression (dieses Gen ist sowohl an der Verbreitung von Antibiotikaresistenz- als auch Virulenzgenen beteiligt) zeigen. Zudem könnte eine bewußte Steuerung der Intensität und Reihenfolge der hervorgerufenen Stressbedingungen (z.B. Prozessbedingungen der Lebensmittelherstellung) zur Vermeidung einer ungewollten Vorkonditionierung von *E. faecalis* hinsichtlich seiner Virulenz beitragen.

1 Introduction

1.1 The dual nature of *Enterococcus faecalis*

Enterococci are lactic acid bacteria (LAB) that are commonly associated with the human digestive tract as commensal organisms and found ubiquitously in the environment. Although enterococci are used in food and feed applications, the dual nature of *Enterococcus faecalis* presents a problem for food safety. On the positive side, these organisms, like others in the LAB group, are used in food fermentations, as probiotics, and to produce bacteriocins against harmful foodborne bacteria. They have a long history of use in the production of Mediterranean-style artisenal cheeses and sausages either as starter cultures or in natural fermentations, and impart unique organoleptic qualities to the final products through citrate metabolism, proteolysis and lipolysis (Foulquie Moreno *et al.*, 2006). Concentrations of enterococci in these products can range from 10^5 to 10^7 CFU/g in fully ripened cheeses (Franz *et al.*, 2003) and from 10^4 to 10^5 CFU/g in slightly fermented sausages (Martin *et al.*, 2005).

Enterococci have also shown beneficial use as probiotics, which by definition are "live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). Studies of potential probiotic strains have demonstrated that these organisms strengthen the immune system, reduce inflammation, and may even be indirectly involved in reducing the incidence of colon cancer (Mercenier *et al.*, 2003). One such human probiotic *E. faecalis* strain currently sold on the market is Symbioflor 1, although *E. faecalis* and *E. faecium* probiotic strains are also available as supplements for animals (Foulquie Moreno *et al.*, 2006).

Finally, enterococci produce antibacterial molecules (bacteriocins) which inhibit the growth and survival of established foodborne pathogens in food products and provide an additional food safety measure. One frequently studied bacteriocin produced by *E. faecalis*,

AS-48, significantly reduced *Listeria monocytogenes* to levels below the detection limit (100 CFU/g) in sausages after 9 days (Ananou *et al.*, 2005), and eliminated the presence of *Bacillus cereus* in milk after 72 hours of co-incubation with an AS-48 producing *E. faecalis* strain (Munoz *et al.*, 2004). Several other *E. faecalis* bacteriocin molecules have also been identified (see Folquie Moreno *et al*., 2006 for a complete review), and function by forming pores in the cytoplasmic membrane of target bacteria, which results in the disruption of the transmembrane potential and pH gradient and the eventual demise of the bacteria (Cleveland *et al.*, 2001).

Unfortunately, the many beneficial contributions of enterococci to human and animal health are offset by the large number of nosocomial infections attributed to these organisms. *E. faecalis* and *E. faecium* are the third most common cause of hospital-acquired infections, which include those of the bloodstream, central nervous system, urinary tract and heart (Giraffa, 2002). Studies conducted to examine the factors contributing to these types of infections have implicated age, a repressed immune system, and long term treatment with antibiotics in the development of infection, although the route of infection was not described. While a low percentage of these infections are fatal and are compounded by the pre-existing immunodeficient condition of the patients (Sakka *et al.*, 2008), these infections are difficult to treat due to the many innate and acquired antibiotic resistances of the infecting enterococcal strains. Innate resistances already exist within the chromosome of the enterococci, and include resistances to β-lactams, clindamycin, fluoroquinolones, and low concentrations of aminoglycosides (Cetinkaya *et al.*, 2000; Foulquie Moreno *et al.*, 2006; Franz *et al.*, 2003). On the other hand, acquired resistances are obtained through the uptake of mobile genetic elements such as plasmids and transposons (Franz *et al.*, 2003), and can include a number of various resistances, of which vancomycin is a top concern. This is due to the fact that vancomycin resistant enterococci (VRE) are also resistant to all other anti-enterococcal drugs,

which limits treatment options for these types of infections (Cetinkaya *et al.*, 2000; Foulquie Moreno *et al.*, 2006; Franz *et al.*, 2003). Since enterococci have a high propensity for genetic exchange with conjugation rates as high as 10^{-1} to 10^{-2} transconjugants per donor cell (Huycke *et al.*, 1992; Waters *et al.*, 2004), antibiotic resistances, including that to vancomycin, can easily and quickly be spread through a population.

This is of special concern for food safety. Since enterococci are common contaminants of foods from animal origin, VRE strains could easily come into contact with an apparently "safe" starter or probiotic strain and pass on genes for antibiotic resistance, which would then be consumed and exposed to a second population of organisms in the host gastrointestinal tract, perpetuating the spread of antibiotic resistance genes. Additionally, there is always the danger that foodborne bacteria with a well-documented history of pathogenicity, including *L. monocytogenes*, *Escherichia coli* O157:H7, and *Staphylococcus aureus*, could acquire these antibiotic resistances and cause an infection that is difficult to eliminate. (Cocconcelli *et al.*, 2003) demonstrated that the transfer of a plasmid containing a vancomycin resistance gene between a donor *E. faecalis* strain and a food *E. faecalis* strain could occur during both sausage and cheese fermentations, with the highest frequency (10^{-3} transconjugants/recipient cell) observed in sausages. Additionally, (Mater, 2008) recently showed that the transfer of vancomycin resistance can also take place in the mouse intestine between enterococci and a "safe" *Lactobacillus acidophilus* commercial strain, accentuating the larger medical implications that the use of enterococci in foods can have on problems with other bacteria acquiring antibiotic resistances.

The dual nature of *E. faecalis* has driven authorities in both Europe and the United States to take a closer look at current established guidelines regarding the use of these organisms in starter cultures and as probiotics. While suggestions range from limiting the use of enterococci to strains lacking key antibiotic resistance genes or certain virulence traits

(reviewed below) to banning the use of enterococci altogether as Canada has done for the use of these organisms as probiotics, the fact remains that there is limited information on the mechanisms which contribute to enterococcal virulence. Thus, before informed decisions can be made in regard to the use of enterococci in food, a greater understanding of the *E. faecalis* virulence process must be obtained.

1.2 *Enterococcus faecalis* and the potential for virulence

Enterococcus faecalis possesses the potential to cause foodborne disease. In addition to its use in the defined products above (i.e. fermented cheeses and meats and probiotics) these bacteria are found in a number of other raw, fermented and ready-to-eat food products, including milk, beef, pork and chicken (Chingwaru *et al.*, 2003; Klein *et al.*, 1998), and fruits and vegetables (Abriouel *et al.*, 2007), capers (Perez-Pulido *et al.*, 2006), chicken salad, chicken burgers and carrot cake (Macovei and Zurek, 2007) from a variety of countries. Concentrations of *E. faecalis* in these foods have been relatively low, ranging between 2.3 to 5.3 log/g in capers and between 0.5×10^1 and 7.1×10^2 CFU/g in raw minced meat (Klein *et al.*, 1998), although the potential to multiply exists. In addition to the high incidence of *E. faecalis* in a wide variety of foods, these isolates possess a number of antibiotic resistances, although the majority remains susceptible to vancomycin.

The high incidence of *E. faecalis* found in foods from all stages of production reflects the environmental hardiness of this organism. Enterococci are able to grow in environments of up to 6.5% salt, pHs between 4.0 and 9.6, and temperatures between 10 and 45°C, and can survive for 30 minutes at 60°C (Foulquie Moreno *et al.*, 2006; Giraffa, 2003; Ogier and Serror, 2007), which allows them to survive the many environmental stresses applied for food preservation (like fermentation). These stresses include lowering the water activity by drying or through the addition of water binding agents such as salt and sugar, applying extreme

temperatures, adjusting the pH, using nitrite, or applying high hydrostatic pressure to inhibit or eliminate undesirable microorganisms in food. Enterococci can also grow in the presence of 40% bile salts (Ogier and Serror, 2007) which, when combined with the ability to survive high temperature and low pH stresses, demonstrates why these organisms are ideal for use as probiotics, as they can survive the probiotic preparation process, as well as the environment within the human digestive system in order to exert the desired effect within the intestine.

The ability to survive the environmental stresses of the gastrointestinal tract is additionally enhanced by pre-exposure to sub-lethal stresses, including those found in the food environment. A number of different cross-protections have been reported for *E. faecalis*, although the observations that pre-adaptation to heat (50°C), NaCl (6.5%) and sugar increased survival upon exposure to 0.3% bile salts, and that pre-adaptation to acid (pH 4.8) increased survival upon homologous challenge with acid of a lower pH (3.2; (Flahaut *et al.*, 1996a; Flahaut *et al.*, 1996c; Rince *et al.*, 2000; Rince *et al.*, 2003) are the most relevant for the current stress conditions under discussion. Thus, the environmental stresses applied to inhibit or eliminate unwanted bacteria in food actually increase the ability of *E. faecalis* to survive the transition to the host gastrointestinal tract.

After *E. faecalis* survives the environmental stresses encountered in food as well as the bile salts and low pH stress conditions of the digestive tract, it is capable of translocation across the intestinal epithelium, which is a crucial step in pathogenesis. In a series of studies conducted by Wells et al., *E. faecalis* was observed to concentrate and adhere more prominently in the lower digestive tracts (the cecum and colon in particular) of orally infected mice (Wells and Erlandsen, 1991), with translocation appearing to occur through intestinal epithelial cells to the mesenteric lymph nodes, liver and spleen to cause death in 13% of the infected mice after 14 days (Wells *et al.*, 1990; Wells *et al.*, 1991). While these studies did not identify the mechanisms or genes involved in *E. faecalis* translocation, they indicate that

E. faecalis is able to selectively adhere to and cross the intestinal epithelial barrier in the lower intestinal tract to cause infection and death.

It is interesting to note that many of the criteria used to select bacteria for starter cultures and as probiotics include the same traits which can pre-dispose them to pathogenicity. Fermentations by LAB in general involve the production of organic acids, alcohol and carbon dioxide from the oxidation of carbohydrates (Caplice and Fitzgerald, 1999; Ross *et al.*, 2002) – end products that inhibit the growth of other bacteria to exert a food preservation effect. Since it would be counter-productive for these conditions to affect the fermentation organisms as well, these organisms, like *E. faecalis*, have evolved mechanisms to deal with stresses encountered during fermentation, including low pH and higher temperatures. Some starter cultures are also selected for their ability to produce bacteriocins, which in some cases work against not only prokaryotic but eukaryotic cells as well. Probiotic strains, on the other hand, must be able to survive a number of other food processing stresses, including the ones encountered during fermentation. Additionally, they must survive and proliferate in the conditions within the GI tract (bile and acid), compete with the normal gut microflora, and adhere to or colonize the intestinal epithelium to exert a positive effect (Klaenhammer and Kullen, 1999; Senok *et al.*, 2005). As a normal gut commensal microorganism, *E. faecalis* has evolved mechanisms to survive all these conditions, which makes it ideal for use as a probiotic. However, these traits, as outlined above, enable *E. faecalis* to easily move from food to the GI tract to cause systemic infection.

1.3 The identification of virulence traits

The identification of traits which contribute to the pathogenicity of *Enterococcus faecalis* represents the first step towards understanding the dual nature of this organism. In correlation with the potential route of infection outlined in the previous section, *E. faecalis*

must first survive the stress conditions encountered during food processing and within the host gastrointestinal tract. Following stress survival, this organism must then be able to sense and attach to the intestinal epithelium. Lastly, *E. faecalis* must penetrate the epithelium (i.e. translocate) to travel to other sites within the host. A number of studies have attempted to identify these virulence traits (genes) through comparative studies which looked for genetic differences between populations of *E. faecalis* isolated from various origins (i.e. from healthy individuals, clinical cases, and food), or by looking for genes similar to those already characterized as virulence-related in similar bacteria. This has led to the identification of a number of virulence-associated genes, which are described in further detail below.

1.3.1 Genes involved in stress survival

dnaK and *groEL*

DnaK and GroEL are well characterized classical chaperone proteins encoded by the class I heat shock genes *dnaK* and *groEL*, which stabilize, prevent aggregation of, and (re)fold proteins in response to bacterial stress (Henderson *et al.*, 2006). While these proteins have been extensively characterized in other bacteria, namely *Escherichia coli* and *Bacillus subtilis*, they were only recently identified (Boutibonnes *et al.*, 1993) and characterized at the transcriptional level (Laport *et al.*, 2004) in *Enterococcus faecalis* (Figure 1). In *B. subtilis*, class I heat shock gene expression is controlled by the HrcA/CIRCE system, where HrcA acts as a negative regulator of these genes by binding to a highly conserved CIRCE sequence (controlling inverted repeat of chaperone expression) located in the regulatory regions prior to the *dnaK* and *groEL* operons (Derre *et al.*, 1999; Mogk *et al.*, 1997). In the presence of an environmental stress, like heat shock, there is an increase in the presence of non-native proteins (i.e. misfolded or aggregated) which titrate away the pool of GroE needed for the stabilization or activation of HrcA. This leads to decreased binding between HrcA and the

CIRCE element, and therefore increased expression of the operons inhibited by HrcA/CIRCE (Mogk *et al.*, 1997). A similar system of regulation was characterized by (Laport *et al.*, 2004) in *E. faecalis*. Both *dnaK* and *groEL* operons are preceded by CIRCE elements, with *hrcA* included as the first gene in the *dnaK* operon. The DnaK and GroEL proteins are expressed in response to heat stress (50°C), ethanol, bile salts, NaCl, pH 4.8 and pH 10.5 (Flahaut *et al.*, 1996a; Flahaut *et al.*, 1996b; Flahaut *et al.*, 1996c; Flahaut *et al.*, 1997; Giard *et al.*, 2001; Laport *et al.*, 2004; Rince *et al.*, 2000), and appear to aid in the survival of *E. faecalis* exposed to these stresses.

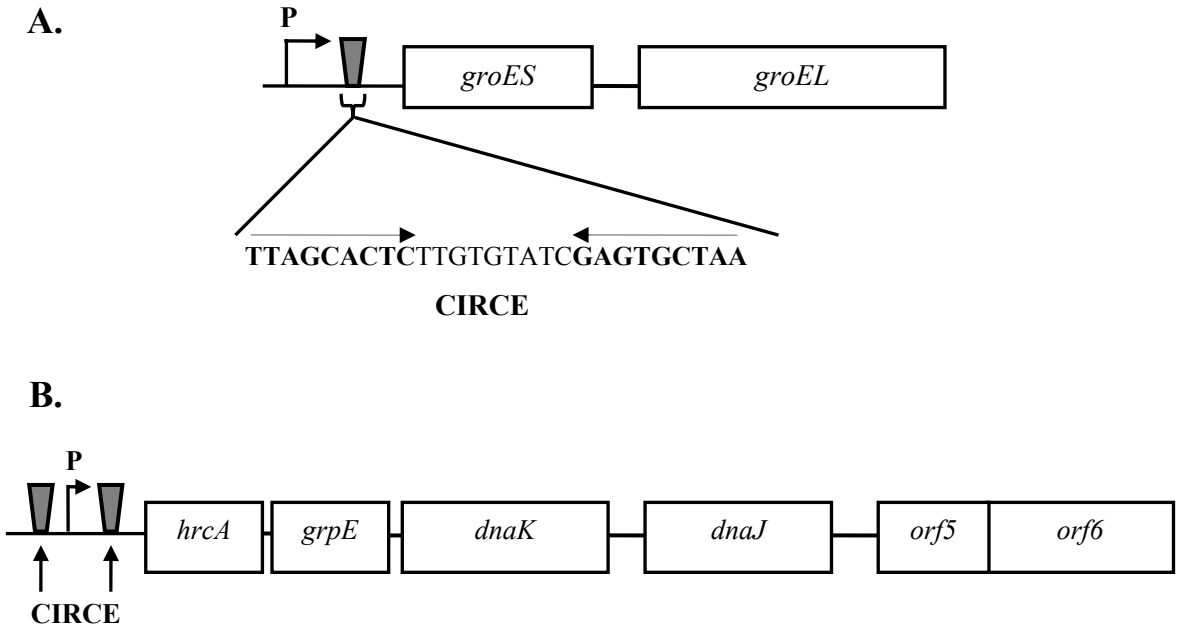

Figure 1. CIRCE sequence and schematic organization of the *groE* (A) and *dnaK* (B) operons of *Enterococcus faecalis*. Adapted from the publication by (Laport *et al.*, 2004).

ctsR, *clpP*, and *clpBCEX*

Similar to the *dnaK* and *groEL* genes discussed in the previous section, the *clp* genes are also activated under stress conditions and function as both chaperones and proteases. While these genes have been studied in a number of other bacteria, their function and regulation has not yet been characterized in *E. faecalis*. The *clp* genes, along with *ctsR*, are part of the class III stress response genes, which include all genes regulated in response to

stress that do not involve the mechanisms used by the class I genes (*hrcA*/CIRCE) or class II genes (alternate sigma factor σ^B). This group includes a number of ATPases (ClpB, ClpC, ClpE and ClpX) belonging to the highly conserved Hsp100 family, which target specific substrates, unfold them, and present them for processing by the proteolytic unit, ClpP (Frees *et al.*, 2003; Frees *et al.*, 2004). In the absence of ClpP, these ATPases also function as molecular chaperones, while ClpP is only able to degrade small peptides in the absence of an associated ATPase. Low GC Gram positive bacteria like *E. faecalis* negatively regulate these genes with *ctsR* (class three stress gene regulator), which is the first gene of an operon including *mcsA*, *mcsB*, and *clpC*. The current model for *ctsR* regulation presented by (Frees *et al.*, 2007)(Figure 2) suggests that McsA, McsB and ClpC form a tertiary complex which inhibits the kinase activity of McsB. When the bacterium experiences a stress, an increase in the concentration of non-native proteins titrates ClpC away from the tertiary complex, similar to the system described in the previous section for control of *dnaK* and *groEL*. This results in the activation of McsB kinase activity by interaction with McsA, and these two proteins bind to CtsR, phosphorylating it and de-repressing its inhibitory activity. Through the process of phosphorylation, CtsR is also labeled for degradation by the ClpCP and ClpEP

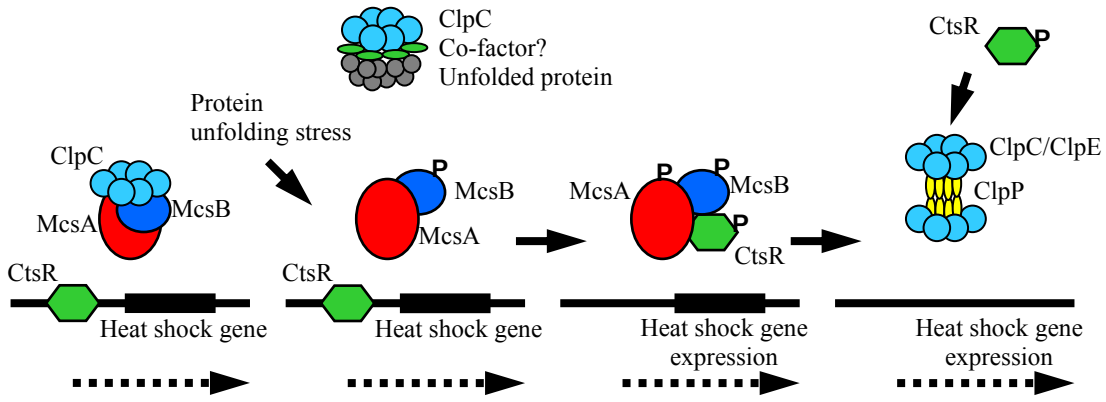

Figure 2. CtsR regulation in Gram positive bacteria. In response to stress, the repressor activity of CtsR is inhibited by McsA/McsB-mediated phosphorylation and subsequent degradation by ClpCP. Details of the model are described in the text. Long arrow indicates high level gene expression, broken arrow indicates low level gene expression and P indicates phsphorylation. Figure and text from (Frees *et al.*, 2007).

ATPase/protease complexes (Kruger *et al.*, 2001). The two other Clp proteins, ClpB and ClpX, appear to involve different mechanisms than the ones already described. ClpB lacks a ClpP binding site and appears to function solely as a chaperone, while ClpX expression is not regulated by *ctsR* and appears to have different functions in different bacteria (Frees *et al.*, 2007).

These stress genes may play a significant role in the virulence process of *E. faecalis*. Not only do they allow *E. faecalis* to cope with the numerous stress conditions encountered in the food processing and GI tract environments, but studies in other organisms, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Listeria monocytogenes*, have demonstrated that the expression of these proteins may play key roles in the virulence process (Chastanet *et al.*, 2004; Frees *et al.*, 2003; Frees *et al.*, 2004; Frees *et al.*, 2005; Henderson *et al.*, 2006; Wang *et al.*, 2007).

General stress protein (*gls24*)

The general stress protein, Gls24, was originally identified in an analysis of *E. faecalis* proteins induced in response to glucose starvation (Giard *et al.*, 1997). Since then, the expression of this protein has been observed in response to other stress treatments, including challenges of 62°C, 20mM H_2O_2, pH 3.7, pH 11.9, 17% ethanol (vol/vol), 0.3% bile salts and 50 mg/ml $CdCl_2$ (Giard *et al.*, 2000; Hartke *et al.*, 1998). Subsequent analyses comparing a *gls24* mutant and its wild type *E. faecalis* strain found that while the mutant had a longer generation time (58 minutes compared to 45 minutes at 37°C) and did not form long coccal chains (80% were pairs or very short chains), its survival under the various stresses, with the exception of 0.3% bile salts, was not impaired (Giard *et al.*, 2000). This implies that Gls24 may play an as yet unidentified role in the *E. faecalis* stress response. However, in both a rat endocarditis model (Nannini *et al.*, 2005) and mouse peritonitis model (Teng *et al.*,

2005), Gls24 appeared to play an important role in the survival and virulence of *E. faecalis in vivo*.

1.3.2 Genes involved in adhesion and attachment

Endocarditis antigen (*efaA*)

EfaA was first identified by antigen analysis of serum from endocarditis patients (Lowe *et al.*, 1995). The gene for this protein encodes a solute binding protein receptor that is part of an operon of genes which includes *efaC*, an ATP-binding protein, *efaB*, a hydrophobic transmembrane protein, and *efaR*, which regulates the expression of *efaCBA* (Low *et al.*, 2003). Together, these genes comprise an ABC-type metal ion transport system specific for Mn^{2+}, whose transcription is induced under conditions of low Mn^{2+} availability. Although the exact mechanism of the involvement of this gene in *Enterococcus faecalis* virulence is unknown, a study conducted in a mouse model to compare virulence of an *E. faecalis efaA* knockout mutant to its wild type found delayed mortality in mice infected with the *efaA* knockout mutant (Singh *et al.*, 1998), which implies that this gene plays a role in *E. faecalis* pathogenicity.

Aggregation substance (*agg*)

The production of aggregation substance (AS) as part of the well-characterized plasmid transfer system in *E. faecalis* has become a point of interest for investigations of its role in *E. faecalis* virulence. AS is a surface molecule that is expressed by plasmid-containing *E. faecalis* cells in response to pheromones produced by cells which lack the plasmid (Galli *et al.*, 1989; Wirth, 1994). Highly conserved sequences in the AS molecule promote cell to cell binding, or aggregation, to enable conjugation. These sequences also appear to promote binding to eukaryotic cells, which initiated investigations into AS involvement in the *E.*

faecalis virulence process. Studies have found that AS promotes binding to fibronectin and other extracellular matrix (ECM) molecules (Isenmann *et al.*, 2002; Rozdzinski *et al.*, 2001), as well as to cultured renal tubular cells (Kreft *et al.*, 1992). Additionally, AS appears to play a role in the adhesion to and internalization by various cell types, including intestinal epithelial cells (Olmsted *et al.*, 1994; Waters *et al.*, 2003b; Wells *et al.*, 2000) and colonic mucosal cells (Isenmann *et al.*, 2000). The ability of *E. faecalis* expressing AS to survive within macrophages was also reported (Rakita *et al.*, 1999; Sussmuth *et al.*, 2000). However, *in vivo* studies conducted to determine the contribution of AS to *E. faecalis* virulence in different animal models have produced conflicting results. While early studies in a rabbit endocarditis model found that AS contributes to increase virulence (Chow *et al.*, 1993; Schlievert *et al.*, 1997; Schlievert *et al.*, 1998), subsequent studies in a mouse model of ascending urinary tract infection (Johnson *et al.*, 2004) and in a rabbit endophthalmitis model (Jett *et al.*, 1998) found that other factors, and not AS, contributed to virulence. Another study suggested that the differences observed between AS-mediated virulence were specific to the model organisms used (Dupont *et al.*, 1998). Thus, the mechanisms behind the involvement of AS in the infection process still remain to be elucidated, and perhaps environments specific to the different hosts play a larger role in AS-mediated virulence.

Enterococcal surface protein (*esp*)

Enterococcal surface protein (Esp) is expressed on the surface of *E. faecalis* cells and was first identified from an *E. faecalis* isolate associated with multiple infections in a hospital ward (Shankar *et al.*, 1999). The association of this virulence factor with infection-derived isolates was significantly higher than with commensal isolates. Due to the unique structure of this protein, which contains motifs commonly found among bacterial surface adhesins that play a role in host ligand binding, and the frequent association of *E. faecalis* with nosocomial

urinary tract infections (UTIs), (Shankar *et al.*, 2001) proposed that Esp is involved in the initial adhesion and colonization event of the pathogenicity process involved in the development of UTIs. Results of this study revealed that Esp did play a role in bladder colonization, and subsequent studies indicate that Esp enhances the ability of *E. faecalis* to form biofilms (Kristich *et al.*, 2004; Tendolkar *et al.*, 2004; Toledo-Arana *et al.*, 2001), which is a key step towards enhancing both survival and pathogenicity in the host environment.

Adhesion to collagen from enterococci (*ace*)

The *E. faecalis* collagen binding surface protein, Ace, was initially described by (Rich *et al.*, 1999) after a search of the *E. faecalis* genome identified a sequence homologous to the *Staphylococcus aureus* virulence factor, Cna. Both of these proteins are MSCRAMMs, or microbial surface component recognizing adhesive matrix molecules, which mediate binding to extracellular matrix components and are anchored to the cell wall via an LP*X*TG motif. In *E. faecalis*, Ace is involved in binding to collagen type I, collagen type IV, collagen type VI, and laminin (Hall *et al.*, 2007; Nallapareddy *et al.*, 2000a; Nallapareddy *et al.*, 2000b; Nallapareddy and Murray, 2006), which are extracellular matrix (ECM) components found in all eukaryotic tissues and commonly used by microbes to promote colonization and initiation of pathogenicity. Although a direct correlation with virulence has not been described for *ace* in an animal model, patient sera from a variety of *E. faecalis* infections (including endocarditis) screened for the presence of Ace-specific IgGs demonstrated strong reactivity against Ace, indicating that it is expressed during *E. faecalis* infection under physiological conditions (Nallapareddy *et al.*, 2000b). Additional studies demonstrated that Ace production is up-regulated at both the transcriptional and translational levels in response to the presence of ECM molecules (Hall *et al.*, 2007; Nallapareddy and Murray, 2006), which implies that *E.*

faecalis senses the presence of these components and modulates *ace* expression in response, although the molecular mechanisms involved in this process have not been characterized.

1.3.3 Genes involved in disruption of the epithelium

gelE and *sprE*

The gene, *gelE*, encodes one of the most studied *Enterococcus faecalis* virulence factors. This gene encodes a metalloprotease capable of degrading fibrin, fibrinogen, collagen, and other biomolecules. It is co-transcribed with *sprE*, which encodes a serine protease, and the products of these two genes appear to work together to degrade misfolded proteins and control chaining (Waters *et al.*, 2003a). Expression of these two genes is controlled by the *fsrABC(D)* genes, of which *fsrA* is constitutively expressed and controls the transcription of *fsrBC(D)* via activation of a separate promoter (Figure 3) (Qin *et al.*, 2001). The C-terminal end of *fsrB* (now tentatively named *fsrD*) produces a peptide pheromone with a lactone ring and is referred to as gelatinase biosynthesis activating pheromone (GBAP) due to its involvement in the induction of *gelE* transcription (Nakayama *et al.*, 2001). GBAP regulates *gelE* transcription in a quorum-sensing manner, and studies have found that about 1,000 GBAP molecules are required per cell for the initiation of *gelE* transcription. A number of studies using *fsrB* and *gelE* mutants in animal models have consistently indicated that these genes play a role in *E. faecalis* virulence (Engelbert *et al.*, 2004; Mylonakis *et al.*, 2002; Qin *et al.*, 2000; Sifri *et al.*, 2002; Singh *et al.*, 2005). Additionally, the purified gelatinase

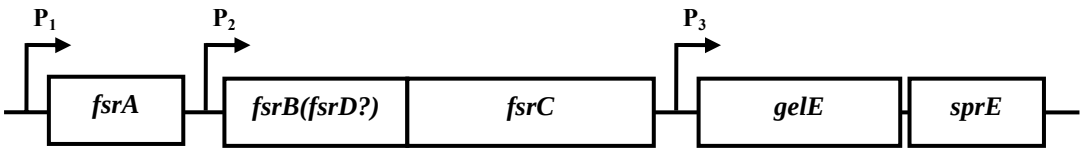

Figure 3. Schematic representation of the *Enterococcus faecalis fsr* and *gelE* operons. Adapted from (Qin *et al.*, 2000).

enzyme was demonstrated to disrupt C3 signaling in serum, which led to disruption of the immune response and evasion of opsonization and membrane attack complex-mediated killing (Park *et al.*, 2007).

The cytolysin operon (*cylL$_L$L$_S$MBAI*)

As with gelatinase, the regulatory mechanisms and contribution to virulence of cytolysin have been extensively studied. This operon includes genes which contribute to the production, processing, and eventual excretion of two peptide subunits, which are lytically active against both prokaryotic and eukaryotic cells. The large and small

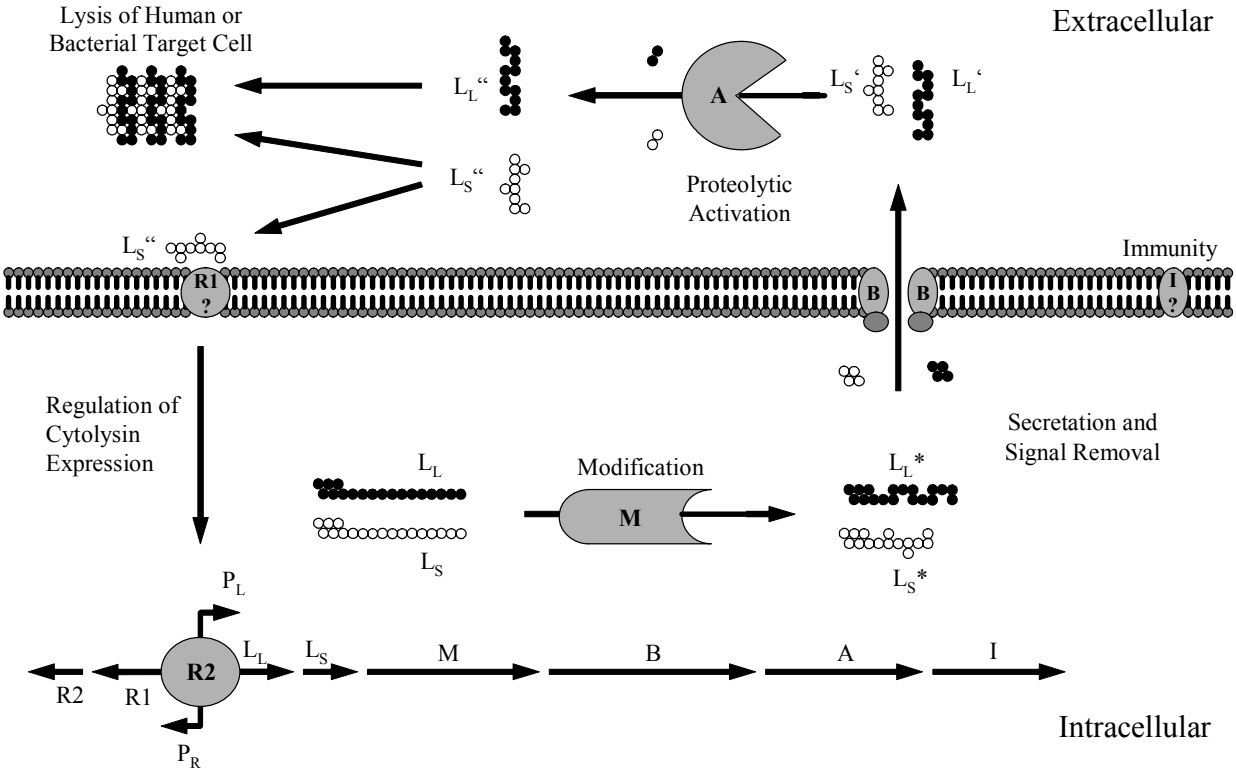

Figure 4. Model for the expression of the cytolysin of *Enterococcus faecalis*. CylL$_L$ and CylL$_S$ are synthesized as 68- and 63-amino-acid precursors, respectively, and then post-translationally modified by the product of the *cylM* gene, generating CylL$_L$* and CylL$_S$*. After modification, both subunits are secreted and processed by CylB, generating CylL$_L$′ and CylL$_S$′. Once both cytolysin subunits have been externalized, an identical six-amino-acid sequence is removed from the N-terminus of each subunit by CylA, generating the active toxin subunits, CylL$_L$″ and CylL$_S$″. The *cylI* gene confers self-protection. Regulation of cytolysin expression is dependent on the products of the *cylR1* and *cylR2* genes, and CylL$_S$″ has been shown to possess signaling activity that results in the autoinduction of the cytolysin operon by a novel quorum-sensing mechanism. Adapted from Coburn and Gilmore (2003).

cytolysin subunits (encoded by *cylL*$_L$ and *cylL*$_S$, respectively) are modified post-translationally by the product of *cylM*, before they are excreted from the cell via an ABC transporter (*cylB*). Once they are outside the cell, the product of *cylA* fully activates the cytolysin subunits, which are prevented from lysing the *E. faecalis* cell by the product encoded by *cylI*, which is an immunity factor (Coburn *et al.*, 2004). Expression of the cytolysin operon is controlled by two genes, *cylR2* and *cylR1*, that repress cytolysin expression in the absence of activated CylL$_S$ (Figure 4) (Haas *et al.*, 2002). The involvement of cytolysin in *E. faecalis* virulence was demonstrated in both mouse and rabbit endophthalmitis models, in which insertional mutants for different genes in the cytolysin operon (non-cytolytic) led to reduced virulence when compared to the wild type (cytolytic; (Ike *et al.*, 1984; Jett *et al.*, 1992).

1.4 Virulence gene regulation

While the identification of genes involved in *Enterococcus faecalis* virulence represents an important step towards understanding the pathogenic nature of this bacterium, knowledge of how environmental factors contribute to the regulation of these genes is of greater importance. Numerous studies have reported that bacteria are able to sense their surroundings and regulate gene expression in response. Since the entire concept of food safety revolves around control of environmental factors which eliminate or limit the growth of pathogenic bacteria in a food system, in order to effectively accomplish this goal, knowledge of bacterial responses to environmental conditions, including the regulation of virulence gene expression, is required.

Knowledge of how virulence genes are regulated has already been demonstrated to play a crucial role in disease treatment with other bacteria. Generally, we tend to treat microbial infections with an arsenal of antibiotics, which target different aspects

of bacterial survival (cell wall, transcription, translation, energy generation or acquisition). However, instances have been discovered in which antibiotic treatment may increase virulence rather than eliminate the bacteria. The bacterium *Pseudomonas aeruginosa* frequently colonizes the lung tissues of cystic fibrosis patients and causes recurring infections due to an inability to treat these infections with a blanket concentration of antibiotics. Instead, concentration gradients of the antibiotic are formed as it is dispersed throughout the bronchiolar system, exposing subsets of the *P. aeruginosa* population to sub-lethal concentrations of antibiotics. (Linares *et al.*, 2006) found that sub-lethal concentrations of tobramycin, ciprofloxacin and tetracycline all induced the formation of biofilm, tobramycin alone induced swimming and swarming, and tetracycline increased *P. aeruginosa* cytotoxicity against macrophages by 4 fold. Thus, the standard method of treatment for bronchiolar *P. aeruginosa* infection promoted, rather than inhibited virulence mechanisms. In another study conducted by (Palazzolo-Ballance, 2008), methicillin-resistant *Staphylococcus aureus* significantly up-regulated the expression of a number of genes involved in toxin and hemolysin production and heme/iron uptake in response to exposure to reactive oxygen species typically produced by human neutrophils. In this case, even defense mechanisms against bacterial infection employed by the human immune system can enhance bacterial virulence. These findings stress the impact that failure to understand the mechanisms behind virulence gene expression can have on bacterial virulence and raise questions about how other conditions may impact virulence gene expression.

The importance of understanding how environmental conditions influence bacterial virulence gene expression is especially important in the field of food safety. Foodborne illness prevention revolves around the control of environmental conditions to either eliminate or control the growth of bacteria. To this end, the pathogen modeling

program (Wilson *et al.*, 2002) was developed along with the Hazard Analysis and Critical Control Points (HACCP) program (Sperber, 1991), to help food companies determine bacterial control parameters for their products. While in these cases, pathogenic bacteria are controlled on a presence/absence basis, the effect of these environmental control conditions on virulence gene expression for the sub-lethally stressed population of bacteria is not considered. The fact that the food environment is not homogeneous and contains protective elements such as fats or oils which can lead to sub-lethally stressed populations of bacteria within the food products should be taken into consideration. Due to the hardy nature of *E. faecalis*, its common association with nosocomial infections, and common presence in foods, before treatment or control options can be properly discussed for *E. faecalis*, an understanding of the conditions which govern virulence gene expression must be gained. Several studies have attempted to look at this regulation in response to different physiological environments. Due to the high association of *E. faecalis* with urinary tract infections and bacteremia, virulence gene expression profiles during growth in urine and serum were investigated (Shepard and Gilmore, 2002). A number of virulence associated genes were up-regulated in these conditions, and expression appeared to be influenced by growth phase and environmental condition. Additionally, the presence of oxygen also had an influence on virulence gene expression, with a number of virulence genes highly expressed under anaerobic conditions consistent with those found in the host (Shepard and Gilmore, 1999). However, this does not present us with a full picture. *E. faecalis* is found ubiquitously in the environment and in foods, and many of these external environmental conditions may precondition this bacterium for virulence before it even enters the host.

1.5 Study Objective

A greater understanding of virulence gene regulation during food processing and as it travels into the GI tract is necessary for its control and to prevent possible foodborne illness caused by *Enterococcus faecalis*. Therefore, virulence gene expression at the transcriptional level in various environments should be characterized in *E. faecalis*, which reflect food processing conditions (temperature, nitrite, high pressure, salt, pH) as well as those found in the host (BHI, pig fecal extract, bile salts, intestinal epithelial cells, mouse model), to gain a better understanding of the conditions which influence virulence gene expression.

2 Materials and Methods

2.1 *Enterococcus faecalis* strains and characterization

2.1.1 *Enterococcus faecalis* strains

Fifteen strains of *Enterococcus faecalis* isolated from various sources, including two genetically modified organisms, were used during the course of this study (Table 1). *E. faecalis* strains 10, 11, A/F2, and 34.5 were kindly provided by Gerhard Reuter (Freie Universität Berlin), and strains OG1RF, TX5264, TX5266 and IL-10 (DNA only) were provided by Dirk Haller (Technische Universität München). The strains OG1RF, TX5264 and TX5266 were originally isolated/generated and described by (Murray *et al.*, 1993; Qin *et al.*, 2001; Sifri *et al.*, 2002; Su *et al.*, 1991), and were used with permission for parts of the current study. Of these strains, five strains of *E. faecalis* (TMW 2.63, 2.629, OG1RF, TX5264, TX5266) were selected for further study. *E. faecalis* TMW 2.63 had the greatest number of virulence genes, TMW 2.629 represented food source isolates, OG1RF has been extensively characterized in a number of other studies conducted by various researchers, and TX5264 and TX5266, which are isogenic mutants of OG1RF, allowed for further characterization of *gelE* regulation in the mouse model and in some cell culture studies.

2.1.2 Growth conditions

Luria-Bertani (LB) Medium:	*Tryptone from casein (enzymatically digested)*	*10.0 g*
	Yeast extract	*5.0 g*
	NaCl	*10.0 g*
	Deionized H_2O	*1000 ml*
	pH	*7.2*

Enterococcus faecalis strains were grown under anaerobic conditions in either LB or Brain Heart Infusion (BHI; Becton Dickinson GmbH, Heidelberg, Germany) medium at 37°C for all experiments. Prior to exposure to all experimental conditions, *E. faecalis*

Table 1. List of *Enterococcus faecalis* strains analyzed during the course of this study. Results from the genotypic and phenotypic analyses are included. All genes listed were detected in the genome (DNA) of the *E. faecalis* strains analyzed, while phenotypic expression of gelatinase (GelE) and cytolysin (Cyl) is denoted by (+) and a lack of expression by (-).

ID	Source	Genotype	Relevant Phenotype
TMW 2.63	Clinical isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *ace*$^+$ *agg*$^+$ *gelE*$^+$ *sprE*$^+$ *cylMBA*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE-, Cyl+
OG1RF	Clinical isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE+
TX5264	TMW 2.622 GMO	TMW 2.622 *gelE* in-frame deletion mutant (bp 252 to 1490), Gel$^-$ Spr$^+$ Rif^r Fus^r	GelE-
TX5266	TMW 2.622 GMO	TMW 2.622 *fsrB* deletion mutant, deletion from bp 79 to 684 of *fsrB*; Gel$^-$ Spr$^-$	GelE-
10	Clinical isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *esp*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE-
11	Clinical isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *agg*$^+$ *esp*$^+$ *ace*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE-
A/F_2	Food	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *agg*$^+$ *ace*$^+$ *gelE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE-
34.5	Food	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *agg*$^+$ *esp*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cob*$^+$ *ccf*$^+$	GelE+
TMW 2.629	Food	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *esp*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE+
TMW 2.630	Food	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cpd*$^+$ *ccf*$^+$	GelE+
IL-10	Mouse isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *agg*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cylMBA*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	
TMW 2.136	Human isolate	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *agg*$^+$ *esp*$^+$ *fsrB*$^+$ *gelE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE+
TMW 2.22^T	Type strain	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *esp*$^+$ *ace*$^+$ *gelE*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE-
TMW 2.354	Duck feces	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE+
TMW 2.355	Duck feces	*clpXP*$^+$ *gls24*$^+$ *efaA*$^+$ *ace*$^+$ *fsrB*$^+$ *gelE*$^+$ *sprE*$^+$ *cpd*$^+$ *cob*$^+$ *ccf*$^+$	GelE+

isolates taken from glycerol stocks were grown overnight (18 h) in the appropriate medium and are referred to as “overnight cultures” within the context of this thesis.

2.1.3 Genetic characterization

2.1.3.1 DNA isolation

Tris-EDTA (TE) Buffer:	*10mM Tris/HCl* *1mM EDTA, pH 8.0*
SDS solution:	*25% (w/v) Sodium dodecyl sulfate in deionized H_2O*
NaCl solution:	*5M NaCl*
Sodium acetate solution:	*3M Sodium acetate, pH 5.2*

DNA was basically isolated according to (Marmur, 1961) with the modification described by (Ehrmann *et al.*, 2003). Briefly, overnight *Enterococcus faecalis* cultures were treated with 0.3 g lysozyme (SERVA Electrophoresis GmbH, Heidelberg, Germany) at 4°C for 18 h before enough SDS solution was added to an end concentration of 2%. After incubation at 60°C for 10 min, enough 5M NaCl was added to bring the final concentration to 1 M before an equal volume of phenol/chloroform (1:1) solution was added. Samples were mixed for 10 min and then centrifuged (5,000 × g, 10 min, 22°C). The upper phase was mixed with an equal volume of chloroform and mixed for 5 min before samples were centrifuged (5,000 × g, 10 min, 22°C). Once again, the upper phase of each sample was saved and then mixed with a volume of sodium acetate solution equal to 1/9 the volume of each sample. A volume of 100% ethanol (-20°C) equal to 2.5 the total sample volume was added to precipitate the DNA before it was collected by centrifugation (5,000 × g, 5 min, 22°C). DNA samples were dried, re-suspended in 200 µl TE-Buffer, and stored at 4°C.

2.1.3.2 Virulence gene analysis using the standard Polymerase Chain Reaction (PCR)

10 × Tris-Borate-EDTA Buffer: *890mM Tris base*
890mM Boric acid
20mM EDTA

DNA was screened for the virulence-associated genes aggregation protein (*agg*), gelatinase (*gelE*), cytolysin (*cylM*, *cylB*, *cylA*), enterococcal surface protein (*esp*), cell wall adhesin (*efaAfs*), and pheromones (*cpd*, *cob*, *ccf*) using the primer sequences from the study conducted by (Eaton and Gasson, 2001). An additional virulence gene, *Enterococcus faecalis* regulator (*fsrB*), was screened using primers from the study of (Shepard and Gilmore, 2002). The other virulence genes adhesin of collagen (*ace*), serine protease (*sprE*), general stress proteins (*groEL, dnaK, ctsR, clpPBCEX, gls24*), and housekeeping genes (*23S*, *adk*, *pyrC*, *gdh*, *gdhA*), were self-designed based upon the published sequences from NCBI. For a complete list of genes, primers and amplicon sizes, see Table 2. PCR amplifications were performed in 25 µl reaction mixtures using 0.5 µl DNA, 16 µM dNTP Mix (Qbiogene, Heidelberg, Germany), 2.5 µl Incubation mix T. Pol with $MgCl_2$ (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA; Qbiogene), 40 pmol of each primer (MWG Biotech AG, Ebersberg, Germany), 0.2 U TaqPolymerase (Qbiogene), and enough water to bring the total volume to 25 µl. Samples were placed in a Primus 96^{plus} thermocycler (MWG Biotech AG) and subjected to an initial denaturation step (94°C for 1 min 30 s), followed by 29 cycles of denaturation (94°C for 30 s), annealing (54°C for 1 min 30 s), and elongation (72°C for 45 s), with a final elongation step of 72°C for 5 min. After amplification, samples were loaded onto a 1.2% agarose gel (SeaKem® LE Agarose, Cambrex Bio Science Rockland, Rockland, ME; 0.5% TBE Buffer), run at 90 V for 1 h, and then subjected to staining with ethidium bromide for 10 min. Results

Table 2. List of *Enterococcus faecalis* primer sequences used for standard PCR and the labeling of probes used for Northern (dot) blot analysis of gene expression. All primers designed during the current study are demarcated by a 1, while the remaining primers with reference number 2 were designed by (Eaton and Gasson, 2001).

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon length (bp)	Reference
23S	CATCCCGCAAGGCTAAATAC	ACCCCACATCCTTTTCCAC	627	1
adk	GACGATTCATCTGTCGCAC	GACTGCCAAACGATTTTTAACC	149	1
pyrC	TCGTGCCATCTAACAAGC	AGAGAACCAGGCTTTACG	706	1
gdh	AAGATGTCCAAGCCAAGC	AGTGTTGTTCCGGTTACG	586	1
gdhA	TGTGAACAGAGGCTATCG	GTGTTGCACATGGTAACG	746	1
groEL	GTTTGTCTGCAACAACTGC	CAAATCGGCGAAACAACG	517	1
dnaK	TTGGTGGTGGTACATTCG	TGACAACGGTGTTACGTC	599	1
ctsR	TTCCTCCTCATAGCTCAAG	CGTCAGATTTAATCGAGGC	449	1
clpP	ACGATCGGTATCACGTTC	AATCATCTCGCGGTGAAC	485	1
clpB	TGAGAGCCGATGTTACTTG	TTAGTTGAAGGCGAACGAG	518	1
clpC	AACCAATCGGTCTTGCTG	AACCTGCTTTAGCACGTG	717	1
clpE	ATATTGCCATCACCAGCAG	CTTTCTCGCCAAATGCAAC	518	1
clpX	CTTTTCCGCACGTTCAAC	TAATAACGGGACCGTTCG	517	1
tuf	CTATGCACACGTTGACTG	ACGTCTGTTGTACGGAAG	789	1
gls24	CCAGAAGATCATGCAATCAAAG	GTTGTCAGAAGCAAATTCACC	423	1
efaAfs	GACAGACCCTCACGAATA	AGTTCATCATGCTGTAGTA	704	2
agg	AAGAAAAAGAAGTAGACCAAC	AAACGGCAAGACAAGTAAATA	1581	2
cpd	TGGTGGGTTATTTTTCAATTC	TACGGCTCTGGCTTACTA	782	2
cob	AACATTCAGCAAACAAAGC	TTGTCATAAAGAGTGGTCAT	1,405	2
ccf	GGGAATTGAGTAGTGAAGAAG	AGCCGCTAAAATCGGTAAAAT	543	2
esp	TTGCTAATGCTAGTCCACGACC	GCGTCAACACTTGCATTGCCGAA	955	2
ace	CCGAATTGAGCAAAAGTTC	AGTGTAACGGACGATAAAG	746	1
gelE	ACCCCGTATCATTGGTTT	ACGCATTGCTTTTCCATC	419	2
sprE	CCTGTCTGCAAATGCAGAAG	CGCCATTGGAATGAACACCA	662	1
cylB	ATTCCTACCTATGTTCTGTTA	AATAAACTCTTCTTTTCCAAC	843	2
cylM	CTGATGGAAAGAAGATAGTAT	TGAGTTGGTCTGATTACATTT	742	2
cylA	TGGATGATAGTGATAGGAAGT	TCTACAGTAAATCTTTCGTCA	517	2

were visualized using an INTAS gel imager (Intas Science Imaging Instruments GmbH, Göttingen, Germany) and a positive result was confirmed by the size of each band (see Table 2 for genes and amplicon sizes).

2.1.4 Phenotypic characterization

2.1.4.1 Gelatinase production

Production of gelatinase was determined by streaking single colonies of all fifteen *Enterococcus faecalis* strains onto LB agar (LB medium, 1.5% (w/v) agarose) containing 3% (w/v) gelatin. Plates were incubated at 37°C for 24 h. Gelatinase production, indicated by zones of opacity around colonies, was visualized after plates were incubated at 4°C for 5 h, as previously described by (Eaton and Gasson, 2001).

2.1.4.2 Cytolysin production

Production of cytolysin was determined by streaking single colonies of all fifteen *Enterococcus faecalis* strains onto blood agar plates (Merck, Darmstadt, Germany). Plates were incubated at 37°C for 24 h. Cytolysin production was indicated by the formation of clear zones around colonies (β-hemolysis).

2.1.5 Growth curves in different test media

Peptone Water for dilutions:	*Tryptone from casein (enzymatically digested)*	*10 g*
	NaCl	*5 g*
	Deionized water	*1L*
	pH 7.2	

2.1.5.1 Luria-Bertani (LB) broth

Growth curves for both *Enterococcus faecalis* TMW 2.63 and OG1RF were determined in LB broth over a 20 h period. Cells were grown overnight in LB medium before fresh

pre-warmed (37°C) LB medium was inoculated with each respective strain at the 1% (v/v) level and incubated without shaking at 37°C. Cultures were sampled after 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 20 h and the appropriate dilutions were plated in duplicate on LB agar plates and incubated at 37°C for 12 h. Optical densities at a wavelength of 590 nm (OD^{590}) were measured at each sampling time with a NovaspecII Spectrophotometer (Amersham Pharmacia Biotech, Piscataway, New Jersey). Results are based upon two separate experiments.

2.1.5.2 Brain Heart Infusion (BHI) broth

Growth curves for *Enterococcus faecalis* TMW 2.63, TMW 2.629 and OG1RF were determined in BHI broth over a 5 hr period. Pre-warmed BHI broth was inoculated at a 1% level with an overnight culture of each of the *E. faecalis* strains. Growth was measured through optical density (OD) measurements only at a wavelength of 590 nm (OD^{590}) at each sampling time with a NovaspecII Spectrophotometer (Amersham Pharmacia Biotech) after 0, 30, 60, 90, 120, 150, 180, 210, 270, 330, 390, 450 and 480 minutes. Results are based upon four separate experiments per bacteria strain.

2.1.6 Determination of sub-lethal stress conditions

2.1.6.1 Growth curves under different stress conditions

Based upon the results obtained in section 2.1.5.2, *Enterococcus faecalis* TMW 2.63, TMW 2.629 and OG1RF were grown in pre-warmed BHI broth inoculated at the 1% level (v/v) from overnight cultures to an OD^{590} of 0.6 (mid/late exponential phase) for all sub-lethal stress experiments. Cultures were then divided equally into 15 mL Sarstedt tubes (SARSTEDT AG & Co, Nümbrecht, Germany), the cells were collected through centrifugation (5000 × g; 7 min), and then re-suspended in fresh BHI broth to

an OD^{590} of 0.1. Enough sterile stress media was added to achieve the following concentrations: $NaNO_2$ (**nitrite stress**; 200 ppm only), NaCl (**osmotic stress**; 0.5, 2.0, 3.5, 4.5, 5.5, 6.0, 6.5%), HCl (**acid stress**; pH 3.9, 4.4, 4.9, 5.9, 6.9, 7.4), and cholic acid:chenodeoxycholic acid (1:1; unconjugated bile salts, Sigma-Aldrich Chemie Gmbh, Munich, Germany; **bile salts stress**; 0, 0.04, 0.06, 0.08, 0.1, 0.12, 0.16, 0.2%). Sterile $NaNO_2$, NaCl and bile salts were prepared by dry sterilization at 120°C for 12 h, while the 1M HCl solution was prepared from a stock solution with sterile deionized H_2O. Cell growth was determined indirectly by measuring the OD^{590} of cell suspensions calibrated against a sterile BHI broth control. Growth rates were determined from OD^{590} changes recorded every 30 min over an 8 h time period. For the chemically modified (NaCl, HCl and bile salts) stress media, measurements were made automatically with a SpectraflourII microtitre plate reader (Tecan, Crailsheim, Germany) at a constant temperature of 37°C. **Heat stress** growth curves were determined manually for the same sampling period and times, and OD^{590} measurements were made with a NovaspecII Spectrophotometer (Amersham Pharmacia Biotech) for cultures incubated at 25, 30, 37, 45, 50, 55 and 60°C.

For the determination of changes in growth under **high hydrostatic pressure (HHP)**, direct cell counts were used instead of OD measurements. This was due to the problems that the extra stresses from obligatory compression and decompression phases of high pressure treatment might exert on the bacteria. Bacteria cultures (OD^{590} of 0.1) were sealed in sterile air-tight plastic pipettes and pressurized for 8 h. Pressures used for each 8 h period were 0.1, 50, 80 and 100 MPa, and the pressurization fluid (water) was maintained at a constant temperature of 37°C. The time to establish pressure as well as the time for decompression was 30 s. After each pressurization period, samples were taken and the appropriate dilutions plated in duplicate on LB Agar plates

according to the standard plate count method (SPC). After an incubation period of around 24 h, the number of colonies per plate was counted. The limits of detection were between 30 and 300 CFU per plate. Results for all experiments are based on three or more experiments.

2.1.6.2 Definition of sub-lethal stress

For reasons of comparability in terms of the effect of different stress conditions on *Enterococcus faecalis* gene expression, sub-lethal stress conditions were defined as the intensity of stress necessary to decrease the growth rate to 10% of the maximum growth rate reached under optimal growth conditions (Scheyhing *et al.*, 2004). This definition of sub-lethal stress is based upon the results of prior studies, which found that this point is associated with the most pronounced variation in gene expression for LAB. Optimal growth conditions were defined as 37°C, no $NaNO_2$, 0.1 MPa, 0.5% NaCl, pH 7.4 and no bile salts. A sub-lethal stress which affects growth to such an extent (10% growth rate reduction) was used because cells treated in this manner elicit a strong stress response. Growth rates under the different stress intensities (μ) were compared to the maximum growth rate (μ_{max}) under optimal conditions to determine which stress intensity results in a growth rate reduction to 10% of the maximum.

2.1.7 Cross resistance between different stresses

The ability of *Enterococcus faecalis* TMW 2.63 to resist lethal stresses following pre-adaptation to the sub-lethal stresses determined in the previous section was determined. This bacterial strain was grown from an overnight culture to an OD^{590} of 0.6 (mid/late exponential phase) in pre-warmed BHI broth. The culture was then equally divided into 15 mL Sarstedt tubes (SARSTEDT AG & Co) and stress was induced directly either by

adding sterile $NaNO_2$, NaCl, HCl and bile salts to end concentrations of 200 ppm, 6.8%, 4.1 and 0.08%, respectively, or by heating (51°C) and pressurization (80 MPa) for 1 h. A reference sample in normal BHI broth was also prepared, which was maintained at ambient pressure and 37°C. After application of the first sub-lethal stress, samples from each condition were taken and bacterial cell counts determined using the previously described SPC method. This group of sub-lethally stressed cells, including the unstressed control in BHI broth, was subsequently subjected to a lethal stress with each of the conditions for 15 min before another SPC was determined (Figure 5). Lethal stress parameters were chosen such that the conditions were stressful enough to let non-adapted cells die off quickly and therefore avoid survival of artefacts (i.e. around 2 log reduction of the cell counts), and yet mild enough to avoid killing too many or even all of the sub-lethally stressed cells (0.2% $NaNO_2$, 22% NaCl, pH 1.9, 0.3% bile salts, 60°C, 300 MPa). The level of tolerance to lethal challenges with each stress, or tolerance factor, was calculated by comparing the % viability of stress adapted cells to the % viability of non-adapted cells according to

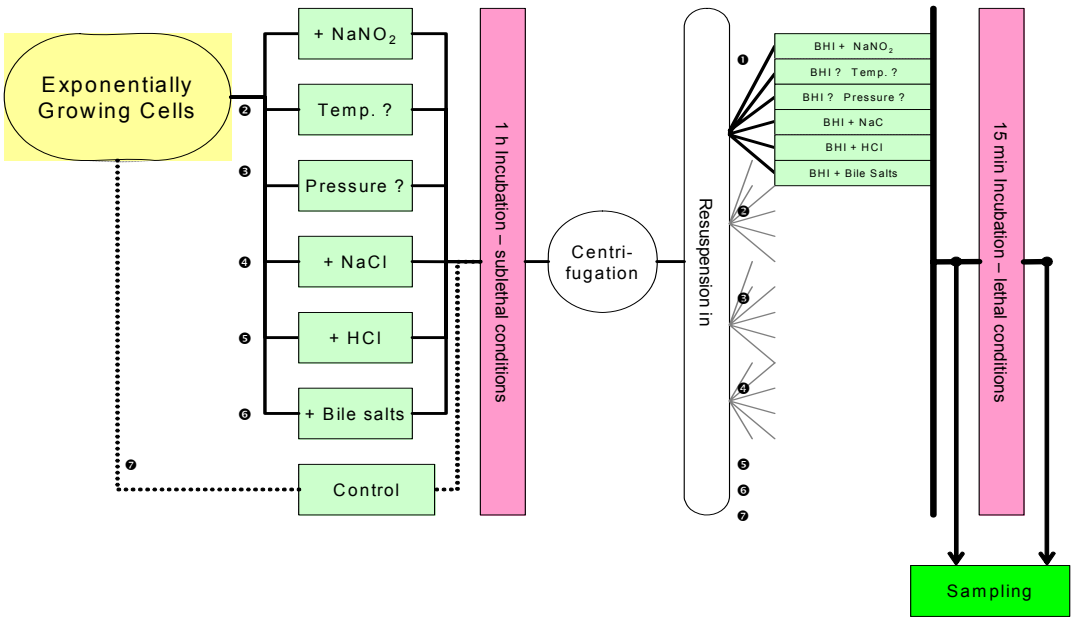

Figure 5. Design of cross-protection experiments and sampling scheme. The dashed line indicates reference, incubated at optimal growth condition during the first application (i.e. no initial sub-lethal pre-stress).

the following equation (Rince *et al.*, 2003):

$$\text{Tolerance factor} = \frac{\%\ \text{viability of stress-adapted cells}}{\%\ \text{viability of non-adapted cells}}$$

Values greater than 1 indicate increased tolerance against the subsequent lethal stress, while a value of 1 indicates that no change in tolerance occurred. Tolerance factors less than 1 indicate that the sub-lethal stress sensitizes *E. faecalis* TMW 2.63 to the subsequent lethal stress. Results are based upon three independent experiments.

2.2 Analysis of virulence gene expression

The manner in which *Enterococcus faecalis* regulates virulence gene expression in response to conditions representative of those encountered during food processing and gut transit was analyzed at the transcriptional level. After *E. faecalis* isolates were subjected to each of the conditions (see sections 2.2.4 and 2.2.5), gene expression at the transcriptional level was analyzed according to the following protocols (see sections 2.2.1, 2.2.2, 2.2.3).

2.2.1 RNA isolation

Multiple protocols exist for the isolation of RNA from bacteria. Due to the fact that *Enterococcus faecalis* is traditionally difficult to work with due its nature as a Gram positive organism, several different techniques were tried in order to optimize the quality and amount of RNA that could be extracted from *E. faecalis* after the various treatments. All buffers and media were prepared with diethylpyrocarbonate (DEPC; Sigma-Aldrich Chemie Gmbh) treated water. DEPC was used to inactivate RNases and prevent RNA degradation.

Diethylpyrocarbonate (DEPC) treated water: *0.1% (v/v) DEPC, 1L deionized H_2O*
12 h at 37°C and autoclaved at 121°C for 20min

2.2.1.1 Beads and Trizol method

RNA was extracted essentially according to the method described by (Shepard and Gilmore, 2002). *Enterococcus faecalis* cells from 10 ml of each medium were collected by centrifugation (5,500 × g, 5 min, 4°C). Pellets were re-suspended in 1.5 ml peQGold RNA Pure (PeQLab, Erlangen, Germany) and transferred to 1.5 ml RNase free screw cap tubes containing 0.25 ml of 106 μm-diameter silicon beads (Sigma-Aldrich Chemie Gmbh) that had previously been heated at 120°C for 12 h to inactivate RNases. The tubes were placed in a water bath and sonicated for a total of 7 min, with manual shaking for 20 s after each minute of sonication to lyse the bacterial cells. After the cells were lysed, tubes were placed on ice for 1 min to allow beads and cell debris to settle out of solution before the supernatant was transferred to 1.5 ml Eppendorf (Eppendorf AG, Hamburg, Germany) safe-lock tubes. The supernatant was clarified by centrifugation (12,000 × g, 10 min, 4°C), collected, mixed with 300 μl of chloroform, and incubated on ice for 15 min. After incubation, samples were centrifuged (12,000 × g, 15 min, 4°C), and then the supernatant was collected and washed with 750 μl chilled isopropanol (4°C). Samples were centrifuged again (12,000 × g, 10 min, 4°C) and the supernatant discarded before washing with 1.5 ml 70% ethanol. Pellets were air dried for 5 to 10 min, re-suspended in 40 μl DEPC-treated water, and frozen with liquid nitrogen for storage at −80°C.

2.2.1.2 Lysozyme and RNEasy Mini Kit (Qiagen)

RNA was isolated according to the protocol provided with the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) with one modification. The bacteria pellet was re-suspended

in 100µl of lysozyme/Tris-EDTA Buffer solution (0.04 g/ml) and incubated at 37°C for 25 min to liberate the RNA before continuing the RNA isolation according to the kit protocol.

2.2.1.3 Lysozyme and Trizol method

RNA was isolated according to the protocol provided with the peQGold RNA Pure isolation reagent (PeQLab) with one modification. The bacteria pellet was re-suspended in 100µl of lysozyme/Tris-EDTA Buffer solution (0.04 g/ml) and incubated at 37°C for 25 min to liberate the RNA before continuing the RNA isolation according to the protocol. Briefly, 1.4 ml peQGold RNA Pure isolation reagent (PeQLab) was added to the lysozyme/Tris-EDTA treated sample, vortexed for 5 s, and then the supernatant was clarified by centrifugation (12,000 × g, 10 min, 4°C). The supernatant was treated with 280 µl of chloroform, vortexed for 15 s, incubated on ice for 5 min, then centrifuged (12,000 × g, 15 min, 4°C). After this, the water soluble fraction was mixed with an equal volume of isopropanol (4°C) and incubated on ice for 15 min before samples were centrifuged (12,000 × g, 15 min, 4°C) to collect the RNA. The RNA was washed twice with 750 µl 70% Ethanol (4°C), air dried for 10 min, re-suspended in 60 µl DEPC treated water, and then incubated at 55°C. Samples were frozen with liquid nitrogen and stored at -80°C.

2.2.2 RNA quality control

All samples were checked for mRNA integrity by loading 4 µl mRNA on a 1.2% agarose gel in 0.5% TBE buffer and running at 90 V for 1 h. mRNA integrity was verified by visualization of the two bands (16S and 23S RNA) with little smearing (Figure 6). Additionally, the mRNA concentration and purity were optically determined

using either a GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, New Jersey), which measured sample absorbencies at wavelengths of 230, 260, and 280, or the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA), which conducted a full spectrum analysis (220-750 nm) of 1.5 µl of each sample. Absorbance ratios for protein (260/280) and organic acid (260/230) contamination were determined using both methods, along with the calculated concentration of total RNA (µg/ml). Pure RNA samples with protein ratios ≥ 1.8 and organic ratios ≥ 1.5 were used for further analysis.

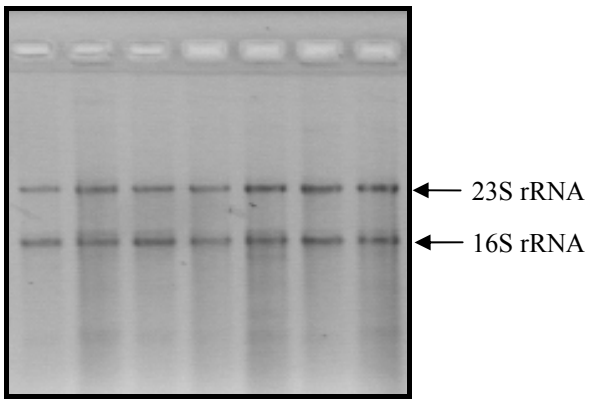

Figure 6. RNA quality and integrity check on a 1.2% agarose gel. 16S and 23S rRNA bands are clearly visible, which indicates that the RNA was not degraded during the extraction process and can safely be used for further analysis.

2.2.3 mRNA quantification

2.2.3.1 Real Time (RT)-PCR

The level of gene expression was determined fluorometrically using SYBR Green I with the Lightcycler® system (Roche Diagnostics GmbH, Mannheim, Germany). SYBR Green dye, which has minimal fluorescence while it is free in solution, selectively binds to the minor groove of double stranded DNA, which significantly enhances its florescence. As the amount of PCR product increases, so does the amount of SYBR Green florescence (measured as a wavelength of 530 nm). The time or cycle number at which the amount of florescence increases above the baseline level is

determined by the amount of cDNA for the given gene in the sample. Samples were examined for differences in gene expression using relative quantification, in which relevant gene expression is normalized to a housekeeping gene using the equation:

$$\text{Expression ratio} = \frac{[E_{\text{gene of interest}}{}^{(\text{Cp control} - \text{Cp sample})}]}{[E_{\text{housekeeping gene}}{}^{(\text{Cp control} - \text{Cp sample})}]}$$

Where Cp is the crossing point at which the level of fluorescence starts to exponentially increase above any background fluorescence (calculated using LightCycler™ Software 5.32, Crossing-Point-Analysis using the Second Derivative Maximum Method), and E (determined by the equation $E = 10^{[-1/\text{slope}]}$) is the efficiency of the reaction. This is in accordance with the method outlined by (Pfaffl, 2001). A master mix containing, per sample, 7.5 µl ABsolute™ QPCR SYBR® Green Mix (ABgene, Surrey, UK), 5.25 µl water, and 0.75 µl primer mix (MWG Biotech AG; containing 500 pmol of each primer), was combined with 1.5 µl of each cDNA sample. This mixture was then centrifuged to deposit samples at the bottom of each Lightcycler® glass capillary tube (Roche Diagnostics GmbH) before being placed into the chamber of the Lightcycler® for real time analysis of PCR amplification. The general PCR program used for all genes was the following:

Program	Target Temperature (°C)	Hold Time	Temperature Transition Rate (°C/s)	Cycles
Polymerase activation	95	15 min	20	1
PCR	a) 95 [1]b) 52 c) 72	15 s 10 s 20 s	20 20 20	35-45
Melting curve analysis	a) 95 [1]b) 62 c) 95	0 s 30 s 0 s	20 20 0.1	1
Cooling	40	30 s	20	1

[1]Depends on primer melting points which can be determined using the equation $T_m = 2°C\ (A+T) + 4°C\ (G+C)$.

A melting curve analysis was conducted to ensure that the detected amplified products were from the target sequence and not from primer dimers, which have low melting temperatures. The housekeeping genes, *23S*, *adk*, *pyrC*, *gdh* and *gdhA* were used as internal controls to which all other virulence gene expression was normalized. Statistical comparison of means was performed using Student's *t* test. All gene expression results are based upon three or more experiments per condition.

2.2.3.1.1 Primer design for RT-PCR

Real time PCR primer sequences for the virulence genes *gls24*, *efaAfs*, *esp*, *ace*, *fsrB*, and $cylL_L$, as well as a housekeeping gene, *23S*, were obtained from (Shepard and Gilmore, 2002). The primer sequence used for the housekeeping gene, adenylate kinase (*adk*), was generated using the MWG online PCR primer design service (MWG Biotech AG). Additional primer sequences for the housekeeping genes *pyrC*, *gdh*, and *gdhA*, as well as genes involved in the stress response, *groEL*, *dnaK*, *ctsR*, *clpP*, *clpB*, *clpC*, *clpE*, *clpX*, and the virulence genes *agg*, *gelE*, *sprE* and *cylB* were self-designed (Table 3). Complete sequences for these genes were obtained from the Pubmed database, accession numbers NC 004668 for all stress genes and *sprE*, and AE 016830 for *adk.*

2.2.3.1.2 Reverse transcription

After the RNA quality was confirmed, a 20 µl RNA aliquot was treated with 5U DNase in 4 µl DNase buffer (Promega, Madison, WI, USA) at 37°C for 1 h before the reaction was stopped with 4 µl of Stop solution (Promega) and incubation at 65°C for 10 min. 10 µl of mRNA was rewritten into cDNA using 2 µg random primers (Promega), 40 µM dNTPs (Qbiogene), 4 µl RT Buffer (Promega), 0.5 U M-MLV Reverse

Table 3. List of *Enterococcus faecalis* primer sequences used for RT-PCR quantification of gene expression. All primers designed during the current study are demarcated by a 1, while the remaining primers with reference number 2 were designed by (Shepard and Gilmore, 2002).

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon length (bp)	Reference
23S	CCTATCGGCCTCGGCTTAG	AGCGAAAGACAGGTGAGAATCC	101	2
adk	GACGATTCATCTGTCGCAC	GACTGCCAAACGATTTTTAACC	149	1
pyrC	TTCAGCCGTATCTGGTAC	AGAGAACCAGGCTTTACG	120	1
gdh	CAAGCATGGTTCCTATGG	AGTGTTGTTCCGGTTACG	103	1
gdhA	GGAATTGATGTGGCGTTAG	GTGTTGCACATGGTAACG	152	1
groEL	CTGTTTCAGTTGCAGCAC	CAAATCGGCGAAACAACG	109	1
dnaK	CAGTTAACCCTGACGAAG	TGACAACGGTGTTACGTC	104	1
ctsR	CGTTGGATGGTAAAACGTG	CGTCAGATTTAATCGAGGC	145	1
clpP	GCAATCACTGAGTTTGCC	AATCATCTCGCGGTGAAC	109	1
clpB	ATCACCGCATCACTAACAG	TTAGTTGAAGGCGAACGAG	104	1
clpC	AATACGGGCAAAACGACG	AACCTGCTTTAGCACGTG	110	1
clpE	CCTAGTAAACCTTGACCAG	CTTTCTCGCCAAATGCAAC	125	1
clpX	TTCTCGGACTGCTTCATC	TAATAACGGGACCGTTCG	154	1
tuf	ACCAGCTACAATCACTCC	ACGTCTGTTGTACGGAAG	129	1
gls24	TAACAGTCGATGGCGGCTTT	CAGCGACTTGTTTTTTACCAACTTC	105	2
efaAfs	TGGGACAGACCCTCACGAATA	CGCCTGTTTCTAAGTTCAAGCC	101	2
agg	GATACAAAGCCAATGTCGTTCCT	GTAAAGAATCGCCACGCTTCAC	102	1
esp	GGAACGCCTTGGTATGCTAAC	GCCACTTTATCAGCCTGAACC	95	2
ace	GGCGACTCAACGTTTGAC	TCCAGCCAAATCGCCTAC	100	2
fsrB	TGCTCAAAAAGCAAAGCCTTATAA	GATGACGAGACCGTAGAGTATTACTGAA	101	2
gelE	GGAACAGACTGCCGGTTTAG	TTCTGGATTAGATGCACCCG	103	1
sprE	CCTGTCTGCAAATGCAGAAG	CTGCCACTTCTTGTCTTCTG	101	1
$cylL_L$	CTGTTGCGGCGACAGCT	CCACCAACCCAGCCACAA	52	2
cylB	ATTGAAGTACGTTGCGCAAG	CCCTTAGTTCTACTAGTGTAC	104	1

Transcriptase, RNase H Minus, Point Mutant (Promega) and 1.5 µl water. mRNA samples were also prepared without reverse transcriptase as a control for DNA contamination. All samples (both with and without reverse transcriptase) were included in a normal PCR (see section 2.1.3.2) with primers for *23S* to ensure that all residual DNA was fully digested with DNase before continuing with the RT-PCR analysis.

2.2.3.2 Northern (dot) blot

20 × SSC Buffer:	*3M NaCl* *0.3M Sodium citrate* *pH 7.0*
10 × MOPS Buffer:	*0.2M MOPS* *50mM Sodium acetate* *20mM EDTA* *pH 7.0*
Denaturing Solution:	*Deionized formamide 0.5ml* *Formaldehyde 162 µl* *1 × MOPS Buffer 100 µl*
10% SDS Solution:	*10% (w/v) SDS in DEPC water*
Low Stringency Wash Buffer:	*2 × SSC Buffer* *0.1% SDS Solution*
High Stringency Wash Buffer:	*0.1 × SSC Buffer* *0.1% SDS Solution*
Maleic Acid Buffer:	*100mM Maleic Acid* *150mM NaCl* *pH 7.5*
Washing Buffer:	*0.3% (v/v) Tween20 (Sigma-Alderich)* *in Malic Acid Buffer*
Blocking Solution I:	*Prepared from dehydrated powder (Roche Diagnostics GmbH)*
Blocking Solution II:	*10% (v/v) Blocking SolutionI in Malic Acid Buffer*
Antibody Solution:	*1/10000 (v/v) Antidigoxigenin-AP (Roche Diagnostics GmbH) in Blocking Solution II*
Detection Buffer:	*0.1M Tris-HCl* *0.1M NaCl* *pH 9.5*

2.2.3.2.1 Northern (dot) blot preparation

A dot blot manifold (Stratagene, Amsterdam, Netherlands) was soaked in a 0.1 M NaOH solution for 45 min and then rinsed thoroughly with sterile deionized water. Denaturing solution consisting of 500 µl deionized formamide (Gerbu Biochemicals, Gaiberg, Germany), 186 µl formaldehyde (Sigma-Alderich Chemie Gmbh), and 100 µl MOPS buffer (Hormann, 2007) was prepared and used to dilute the RNA samples. Samples were incubated at 65°C for 15 min before 20 and 80 µl of each dilution was applied to a positively charged nylon membrane (Amersham Hybond™-N+, GE Healthcare, München, Germany) using a water vacuum pump. Samples were fixed to the membrane for 1 min using ultraviolet light (254 nm), and stored at 4°C until pre-hybridization.

2.2.3.2.2 Preparation of digoxigenin (DIG)-labeled DNA probes

Nonradioactive probes to detect mRNA transcripts from *Enterococcus faecalis* genes significantly induced under the different environmental conditions (see Table 2 for sequences) were prepared using DIG-dUTP (Roche Diagnostics GmbH) incorporation during PCR. A mix of 50 ng DNA, 2 µl Taq Buffer (2.5 M $MgCl_2$, Qbiogene), 0.2 µl of each primer (MWG Biotech AG), 0.4 µl DIG-dUTP, 1.4 µl dNTP (Qbiogene), 16.2 µl PCR water, and 0.4 U Taq polymerase (Qbiogene) was made for each target gene and placed into 0.2 ml thin-walled PCR tubes. A second control sample for each gene was also prepared without DIG-dUTP and water was substituted instead to keep the concentrations of the other reagents consistent. The tubes were placed into a Eppendorf Mastercycler® gradient thermal cycler (Eppendorf AG) and subjected to an initial denaturation step (94°C for 1 min 30 s), followed by 29 cycles of denaturation (94°C for 30 s), annealing (54°C for 1 min 30 s), and elongation (72°C for 45 s), with a final

elongation step of 72°C for 5 min. After amplification, 3 µl from each sample was loaded onto a 1.2% agarose gel (SeaKem® LE Agarose, Cambrex Bio Science Rockland; 0.5% TBE Buffer), run at 90 V for 1 h, and then subjected to staining with ethidium bromide for 10 min. Results were visualized using an INTAS gel imager (Intas Science Imaging Instruments GmbH) and a positive result was confirmed by the size of each band. Efficient incorporation of the DIG-dUTPs produced a band that ran slightly slower than the non-DIG labeled sample (Roche Diagnostics GmbH; Figure 7).

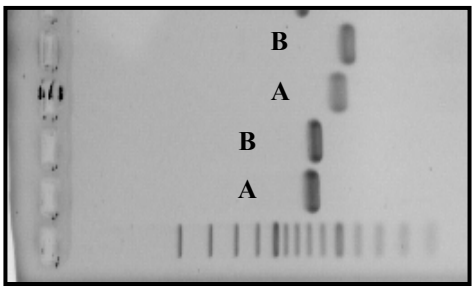

Figure 7. Dioxygenen (DIG) labeled DNA probes for Northern (dot) blot analysis. Probes with efficient incorporation of DIG (A) run slower on a gel compared to samples without DIG (B).

2.2.3.2.3 Hybridization conditions and membrane development

Membranes were pre-hybridized in ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion Applied Biosystems, Darmstadt, Germany) for 30 min at 50°C in a Hybaid hybridization oven (MWG Biotech AG) before 5µl of denatured DIG-labeled probe was added to the buffer. Membranes were then hybridized for 16-24 h at 50°C. After hybridization, membranes were washed two times for 5 min at room temperature with Low Stringency Wash Buffer followed by two 15 min washes at 50°C with High Stringency Wash Buffer. Membranes were then washed for 2 min with Washing Buffer and blocked with Blocking Solution II for 30 min at room temperature. The blocked membranes were then incubated at room temperature for 30 min with Antibody Solution. This was followed by two 15 min washes with Washing Buffer and a 3 min incubation in Detection Buffer. Membranes were then sealed in plastic sheets with

CSPD substrate, ready to use (Roche Diagnostics GmbH) according to the manufacturer's protocol and exposed to x-ray film for 24 h. Films were developed according to the manufacturer's instructions (Kodak, Sigma-Alderich Chemie Gmbh) and scanned for further analysis of spot intensities.

2.2.4 Food preservation and processing conditions

2.2.4.1 Food environment – initial experiments

2.2.4.1.1 Media preparation

Environmental conditions designed to simulate those associated with food processing were prepared. LB broth was used as the basic environmental condition to which virulence gene expression in all other media was compared. Supernatants from *Enterococcus faecalis* strains TMW 2.63 and OG1RF grown in LB broth to late exponential and stationary phases were examined for the ability to change virulence gene expression patterns. Results from these experiments would indicate the presence of molecules involved in quorum sensing. After centrifugation (7,000 × g for 18 min at 23°C), supernatants were collected and the pH was adjusted to 7.0 using 2M sodium hydroxide (NaOH) before sterile filtering (0.22 μm). A liquid meat simulation medium (MSM) was also prepared to represent conditions in sausage in accordance with the components outlined by (Leroy *et al.*, 2005). Finally, pH and salt concentration (osmolarity) were also examined for the ability to influence virulence gene expression. LB broth solutions were prepared and adjusted to pH 7 with 6.5% salt (6.5% NaCl), pH 5 with normal salt (pH5), and pH 5 with 6.5% salt (6.5% NaCl + pH5).

2.2.4.1.2 Culture preparation and sampling

In preparation for the experiments to determine gene expression in the different environments, *Enterococcus faecalis* TMW 2.63 and OG1RF were grown in 20 mL LB broth at 37°C overnight and then re-inoculated into 50 mL LB broth at a 1% (v/v) level. Two cultures of each strain were prepared to represent both late exponential and stationary phase cells (OD^{590} = 0.4 and 0.56, respectively), determined through growth curve analysis in LB broth. Once the cells reached the appropriate OD^{590}, they were collected through centrifugation (6,500 × g, 23°C, 19 min), re-suspended in fresh LB broth, and transferred to 15 mL Sarstedt tubes (SARSTEDT AG & Co.). The cells were again collected through centrifugation and re-suspended in 10 mL of the appropriate environment (i.e. LB, respective supernatant, MSM, 6.5% salt, pH 5, or 6.5% salt + pH 5). Bacterial cells were exposed to each environment for 1 h at 37°C without shaking before mRNA was extracted for gene expression analysis. Results are based on three or more independent experiments.

2.2.4.2 Sub-lethal stresses encountered in the food environment

2.2.4.2.1 Media preparation

Sterile dry $NaNO_2$ and NaCl were prepared by heating the chemicals overnight (12 h) at 120°C before the appropriate amount of each chemical was added to actively growing cultures in BHI broth (see section below). HCl was also sterilely prepared from a stock solution diluted to 1M with sterile distilled H_2O and was added to actively growing cultures in BHI broth to achieve the correct final concentration.

2.2.4.2.2 Culture preparation and sampling

The transcriptional response of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF to sub-lethal stress was determined. Each bacterial strain was grown from an overnight

culture to an OD^{590} of 0.6 (mid/late exponential phase) in pre-warmed BHI broth. Cultures were then equally divided into 15 mL Sarstedt tubes (SARSTEDT AG & Co) and stress was induced directly either by adding sterile $NaNO_2$, NaCl, and HCl to end concentrations of 200 ppm, 6.8%, and 4.1, respectively, or by heating (51°C) and pressurization (80 MPa) for 1 h. A reference sample in normal BHI broth was also prepared, which was maintained at ambient pressure and 37°C. After the 1 h application of each stress, 750 µl samples from each condition were taken and the RNA stabilized with RNAprotect® Bacteria Reagent (Qiagen GmbH) according to manufacturer's instructions. Stabilized samples were frozen with liquid nitrogen and stored at -80°C for further analysis. Results are based on three or more independent experiments.

2.2.5 Physiological conditions associated with the host and gut transit

2.2.5.1 Media environments used to simulate physiological conditions

2.2.5.1.1 Media preparation

Environmental conditions designed to simulate those associated with gut transit were prepared. BHI broth was used due to the association of enterococcal infections of the heart (endocarditis). Porcine fecal extract (PFE) was also prepared to simulate the environment of the lower intestine (colon) by mixing 100 g pig fecal sample from 100 kg pigs with 200 mL distilled water. The fecal sample was suspended evenly in the water followed by centrifugation to eliminate particles, and then sterile filtered through a Millipore Millex GV filter unit (0.22 µm; Millipore, Co., Billerica, MA). LB broth was used as the basic environmental condition to which virulence gene expression in BHI broth and PFE was compared.

Sub-lethal bile salt stress was also investigated due to the involvement of bile salts in the host digestion process. Based on the results from section 2.1.6, 0.08% sterile unconjugated bile salts (Sigma-Alderich Chemie Gmbh) in a mix of cholic acid sodium salt:deoxycholic acid sodium salt (1:1) were dry sterilized at 120°C for at least 12 h before the appropriate amount of the dry bile salts was added to an actively growing culture of *Enterococcus faecalis* in BHI broth to achieve the selected concentration. Bile salt sub-lethal stress gene expression was calculated relative to that observed in BHI.

2.2.5.1.2 Culture preparation and sampling

Enterococcus faecalis strains TMW 2.63 and OG1RF were prepared and sampled according to the same protocol outlined in section 2.2.4.1.2 for the media experiments with LB broth, BHI broth, and PFE. Results are based on three independent experiments.

For the sub-lethal bile salts stress, *E. faecalis* strains TMW 2.63, TMW 2.629 and OG1RF were prepared and sampled according to the same protocol outlined in section 2.2.4.2.2. Results are based on three independent experiments.

2.2.5.2 Intestinal epithelial cells (IEC): an *in vitro* model of *Enterococcus faecalis* infection

10 × Phosphate Buffered Saline: *15mM KH_2PO4*
20mM Na_2HPO_4
27mM KCl
1.37M NaCl

Mode K cell culture medium: *Dulbecco's Modified Eagle Medium (DMEM), 4500mg/l glucose*
10% (v/v) Inactivated Fetal Calf Serum
200mM L-Glutamine
4 mL Antibiotic/antimicotic solution

HT 29 cell culture medium: *Dulbecco's Modified Eagle Medium (DMEM), 4500mg/l glucose*
10% (v/v) Inactivated Fetal Calf Serum
1% (v/v) Non-essential Amino Acids
1% (v/v) L-Glutamine
1% Antibiotic/antimicotic solution

2.2.5.2.1 IEC culture preparation

The human colon adenocarcinoma cell lines HT-29 P (undifferentiated) and HT-29 MTX (differentiated), as well as the mouse intestinal epithelial cell line Mode-K (provided and cultured by Dirk Haller and Micha Hoffmann, respecively; Bifunktionalität der Lebensmittel, Technische Universität München) were grown in a humidified 5% CO_2 atmosphere at 37°C to 90-100% confluence in 250 ml CELLSTAR® Filter cap cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) according to standard cell culture techniques (Ruiz *et al.*, 2005). During growth to confluence, the appropriate cell culture medium was used as an incubation medium (see compositions above). Prior to stimulation with bacteria, the cell cultures were washed three times with 10 ml sterile PBS to remove antibiotics.

2.2.5.2.2 Bacterial culture preparation and sampling

Enterococcus faecalis strains TMW 2.63 (clinical), TMW 2.629 (food), OG1RF (well characterized, clinical), TX5264 (OG1RFΔ*gelE*) and TX5264 (OG1RFΔ*fsrB*) were all cultivated in BHI medium at 37°C. Bacteria were grown overnight in BHI medium before fresh pre-warmed (37°C) BHI medium was inoculated with each respective strain at the 1% (v/v) level and incubated without shaking at 37°C. Cultures were grown to an optical density of 0.2 (early mid-exponential phase) at a wavelength of 590 nm (OD^{590}) and then centrifuged (3,500 × g, 10 min, 21°C). Bacteria were re-suspended in the respective cell culture media described above (D-MEM high glucose,

Gibco Invitrogen) and then used to stimulate three intestinal epithelial cell lines (HT-29 P, HT-29 MTX, and Mode-K) at a multiplicity of infection (MOI) of 100:1. Separate aliquots from the same bacterial culture suspensions in cell culture medium without the addition of cell cultures were included for comparison. Bacteria were incubated in a humidified 5% CO_2 atmosphere at 37°C in their respective environments for 30 min before samples were taken for analysis. RNA was immediately stabilized by the addition of RNAprotect® Bacteria Reagent (Qiagen GmbH) according to the manufacturer's directions and stored at -80°C until RNA analysis was conducted using the RNA extraction protocols described in sections 2.2.1.2 and 2.2.1.3. Results are based upon three or more experiments per bacterial strain.

2.2.5.2.3 Determination of bacterial cell counts

Bacterial counts of cultures before and after IEC stimulation were determined to ensure that differences in gene expression were not due to differences in bacteria numbers, as *Enterococcus faecalis* uses several quorum-sensing mechanisms for virulence gene regulation. Appropriate dilutions were plated in duplicate on LB agar plates and incubated at 37°C for 12 h.

2.2.5.3 Mouse model: an *in vivo* model of *Enterococcus faecalis* infection

2.2.5.3.1 Exploratory mouse study

An exploratory mouse study was conducted by Micha Hoffmann and Dirk Haller (Biofunktionalität der Lebensmittel, Technische Universität München) to determine the effect of a clinical *Enterococcus faecalis* strain (OG1RF) on the development of colitis in interleukin-10 gene deficient (IL-$10^{-/-}$) mice. *E. faecalis* OG1RF was monoassociated with both germ-free IL-$10^{-/-}$ 129 SvEv TAC mice and germ-free 129 SvEv TAC wild

type (WT) mice by gastric lavage according to the protocol in (Ruiz *et al.*, 2005; Shkoda *et al.*, 2007), with germ-free mice as a control. After 7, 14 and 98 days of monoassociation, sections of the intestinal tract were collected, fixed in 10% neutral buffered formalin, and embedded in paraffin for histological analysis to determine the level of inflammation associated with this *E. faecalis* strain (Ruiz *et al.*, 2005; Shkoda *et al.*, 2007). The intestinal contents were also collected and preserved at -80°C for analysis of *E. faecalis* virulence gene expression in our laboratory. Mouse intestinal contents were suspended in 3 ml TE Buffer and then centrifuged (800 × g, 4°C, 11 min) to separate intestinal debris from *E. faecalis* cells. The supernatant was collected and mixed with an additional 1 ml TE Buffer before samples were centrifuged (800 × g, 4°C, 11 min). Again, the supernatant was saved, and remaining cells in the supernatant were collected by centrifugation (3,000 × g, 4°C, 5 min). The collected *E. faecalis* cells were re-suspended in 1.5 ml peQGold RNA Pure isolation reagent (PeQLab) and RNA was isolated and re-written into cDNA according to the protocols outlined in sections 2.2.1.1 and 2.2.3.1.2. Virulence gene expression was determined on the basis of the presence or absence of amplified product following normal PCR (see section 2.1.3.2) using primers listed in Table 2. Results from this study were used to direct the additional mouse experiments outlined in the following sections.

2.2.5.3.2 Follow-up study: Bacterial monoassociation and mouse sample preparation

Based upon the results from the previous section, which indicated that the *Enterococcus faecalis* metalloprotease, GelE, may contribute to colitis in the mouse (see results section), mouse studies using *E. faecalis* isogenic mutants for two genes involved in the production of gelatinase were conducted by Micha Hoffmann and Dirk Haller according to the protocols outlined by (Ruiz *et al.*, 2005) and (Shkoda *et al.*,

2007). Briefly, germ-free IL-10$^{-/-}$129 SvEv TAC mice and their germ-free 129 SvEv TAC wild type counterparts (WT) were separately monoassociated with either *E. faecalis* OG1RF or one of two *E. faecalis* OG1RF isogenic deletion mutants for *gelE* (TX5264) and *fsrB* (TX5266) by gastric lavage. Germ-free mice were used as controls. After 1 and 15 weeks of monoassociation, sections from the intestinal tract were taken and fixed in 10% neutral buffered formalin and then paraffin for histological analysis according to the protocol described by (Ruiz *et al.*, 2005) and (Shkoda *et al.*, 2007). Intestinal contents from each mouse were collected and stored at -80°C for bacterial analysis in our lab.

2.2.5.3.3 Follow-up study: Determination of intestinal bacterial counts in WT and IL-10$^{-/-}$ mice

Intestinal bacterial counts were determined using 100 µl of the supernatant from re-suspended mouse intestinal samples (see section 2.2.5.3.5) serially diluted in peptone water (10 g peptone from casein, 5 g NaCl). 100 µl of each dilution was plated in duplicate on LB agar plates and incubated at 37°C for 24 h before colony counts were made. Results are based upon two groups of six mice and are reported as the number of log colony forming units per gram of intestinal content (log CFU/g).

2.2.5.3.4 Follow-up study: Bacteria verification from colonized WT and IL-10$^{-/-}$ mice

A single representative colony from each mouse fecal sample was taken from the LB plates used to determine the fecal bacterial counts. All colonies had the same shape, size and morphology at sampling time. These colonies were grown overnight at 37°C in 12 ml BHI, and the DNA was isolated according to the procedure in section 2.1.3.1. Expected isolates from the mouse fecal samples were *Enterococcus faecalis* OG1RF

(wild type), and two OG1RF deletion mutants – TX5264 (Δ*gelE*) and TX5266 (Δ*fsrB*). PCR using primers designed to amplify parts of the *E. faecalis 23S* and *sprE* genes, as well as the internal deleted regions of the *gel*E and *fsrB* genes (Tables 2 and 3, respectively), was conducted to confirm the genotypic profile for the bacteria isolated from the mouse fecal samples.

2.2.5.3.5 Follow-up study: Preparation of mouse intestinal samples for RNA analysis

Cecal and colonic intestinal contents from germfree wild type (WT) and IL-10 gene knockout (IL-$10^{-/-}$) mice were weighed and suspended in enough sterile PBS (4°C) to dilute samples 1:10 (wt/vol). Samples were centrifuged for 5 min at 200 × g at 4°C to separate fecal debris from bacteria. After 100 µl of the supernatant was taken to determine fecal bacterial counts (see section 2.2.5.3.3), the remaining supernatant was divided into aliquots of 500 µl, placed into 1.5 ml Eppendorf (Eppendorf AG) safe-lock tubes and mixed with 1 ml of RNAprotect® Bacteria Reagent (Qiagen GmbH) in accordance with manufacturer instructions. Samples were centrifuged (7 min at 12,000 × g at 4°C) to collect the bacterial pellets, which were then frozen with liquid nitrogen and stored at -80°C until RNA was isolated according to the protocol discussed earlier (section 2.2.1.3).

3 Results

3.1 *Enterococcus faecalis* strains and characterization

3.1.1 Genotypic characterization

There were no significant differences in the number of virulence genes found in *Enterococcus faecalis* isolates from clinical, food, or fecal origin (Table 1). All strains, including those isolated from food, possessed between nine and fifteen virulence genes, with the most common genes including *gls24* and *efaA* (100% of the isolates) and the pheromones (*cpd*, *ccf*, *cob*).

3.1.2 Phenotypic characterization

3.1.2.1 Gelatinase production

All *Enterococcus faecalis* strains that possessed genes for both *fsrB* and *gelE* produced gelatinase (Table 1). Zones of clearing around colonies ranged from 7 to 10 mm in diameter after 18 h incubation at 37°C. While only one clinical isolate produced gelatinase, three of four food isolates and four of five fecal isolates produced gelatinase. Of the *E. faecalis* strains chosen for further investigation, TMW 2.629 and OG1RF produce active gelatinase while TMW 2.63 and the two OG1RF mutants, TX5264 and TX5266, do not (Figure 8).

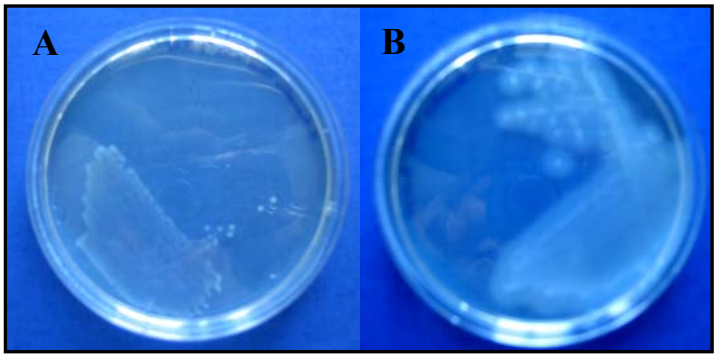

Figure 8. Phenotypic test for gelatinase expression. *Enterococcus faecalis* TMW 2.63, which is $gelE^+$ but $fsrB^-$, does not produce gelatinase (A) while OG1RF, which is $gelE^+$ and $fsrB^+$, does produce gelatinase (B), which is indicated by the opaque halo around growing colonies.

3.1.2.2 Cytolysin production

Only *Enterococcus faecalis* 2.63, a clinical isolate, produced active cytolysin, which was visualized by zones of clearing around colonies consistent with β-hemolysis (Figure 9). This is consistent with the genotypic profile of this isolate, which possessed all *cyl* genes examined.

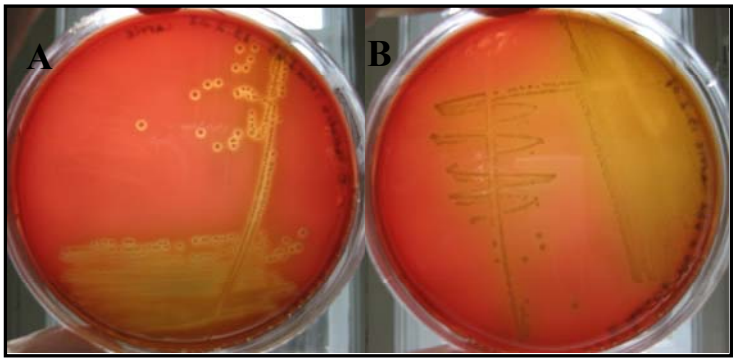

Figure 9. Phenotypic test for cytolysin expression. *Enterococcus faecalis* TMW 2.63 demonstrates β-hemolysis (A), in which a clear zone is observed around growing colonies due to the lysis of red blood cells in the agar. OG1RF demonstrates α-hemolysis (B), or a lack of blood cell lysis around growing colonies, which has a greenish tint rather than clear zones.

3.1.3 Growth curves in different test media

3.1.3.1 LB broth

Growth of both *Enterococcus faecalis* TMW 2.63 and OG1RF was determined in LB broth over a 20 h period. At each sampling time, the OD^{590} was determined and correlated to the bacterial cell count based upon serial dilutions on LB agar plates. While both strains grew readily in LB broth, *E. faecalis* TMW 2.63 grew slightly faster and began the transition to exponential phase between 1.5 and 2 h compared to between 2 and 3 h for *E. faecalis* OG1RF. Growth for TMW 2.63 peaked at an OD^{590} of 0.7, while OG1RF was lower at an OD^{590} of 0.55 (Figure 10). Results of these experiments were used to select bacteria in the appropriate growth phase for the virulence gene expression experiments.

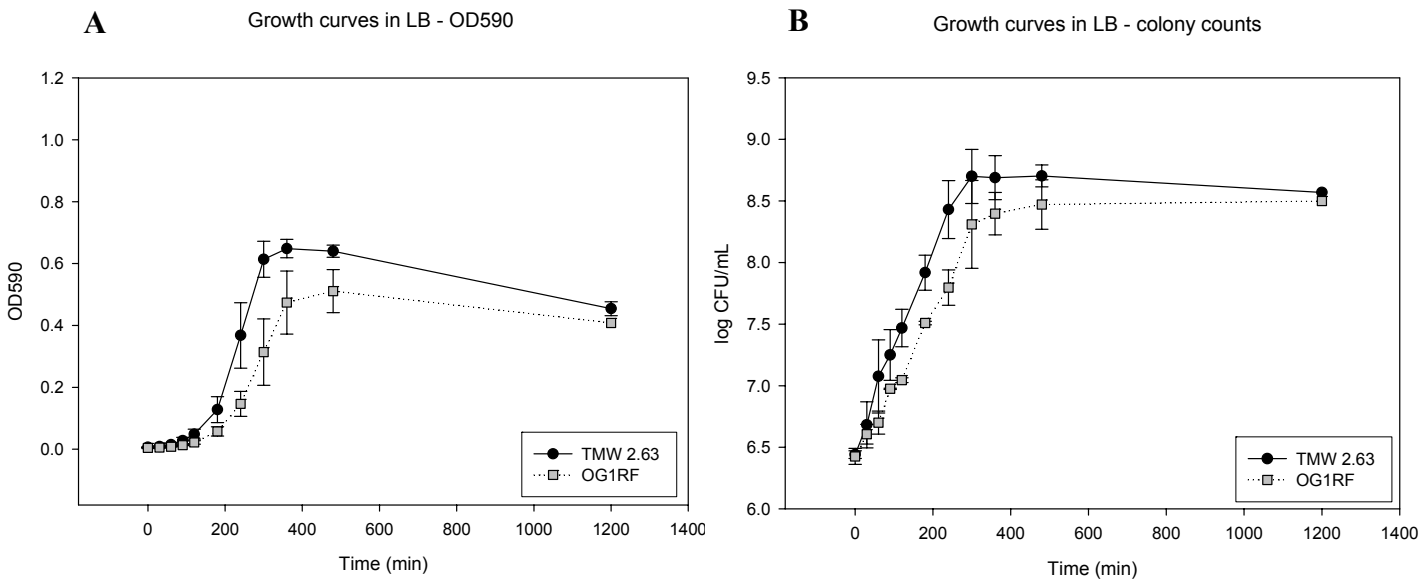

Figure 10. Growth curves of *Enterococcus faecalis* TMW 2.63 and OG1RF in LB broth. Cell growth was determined by (A) cell density using optical density measurements at a wavelength of 590 (OD^{590}) and (B) the standard plate count method at various timepoints. Results using the standard plate count method are reported as the log of the number of colony forming units (CFU) per mL of sample (log CRU/mL).

3.1.3.2 BHI broth

Growth of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF was determined in BHI broth over a 5 h period. At each sampling time, the OD^{590} was determined. All three strains grew readily in BHI broth, although *E. faecalis* TMW 2.629 grew slightly faster than *E. faecalis* TMW 2.63 and OG1RF, with exponential phase starting after

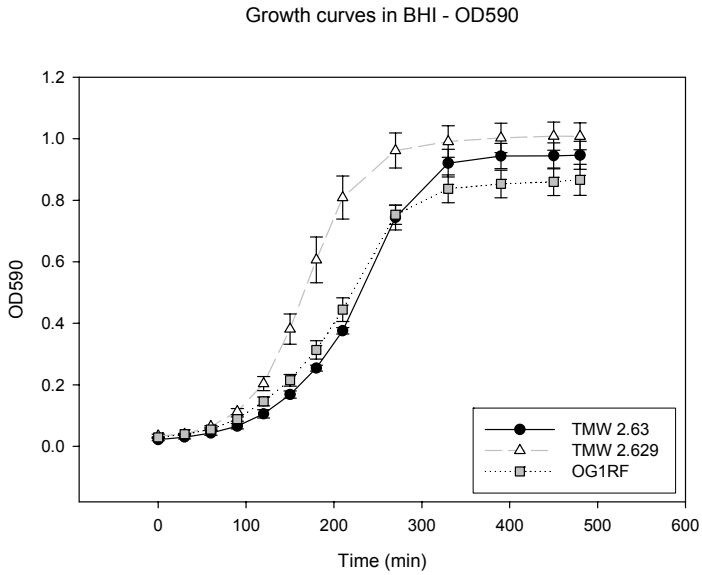

Figure 11. Growth curves for *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF in BHI broth. Cell growth was determined using optical density measurements at a wavelength of 590 (OD^{590}) over a period of 5 h.

about 2 h compared to about 2.5 h for TMW 2.63 and OG1RF. Mid-exponential phase occurred at an OD^{590} of 0.6 for all three strains, with the entry to stationary phase at an OD^{590} between 0.85 and 1.0 (Figure 11). The result of these experiments was used to select bacteria in the appropriate growth phase for the analysis of sub-lethal stress responses.

3.1.4 Growth curves under stress conditions: Determination of sub-lethal stress

3.1.4.1 Heat stress

The growth of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF at various temperatures (heat), including 25, 30, 37, 45, 50, 55, and 60°C, was determined in BHI broth over a period of 8 h. All three strains responded similarly under these conditions, and a reduction in growth rate to 10% of the maximum growth rate occurred at 51°C (Figure 12).

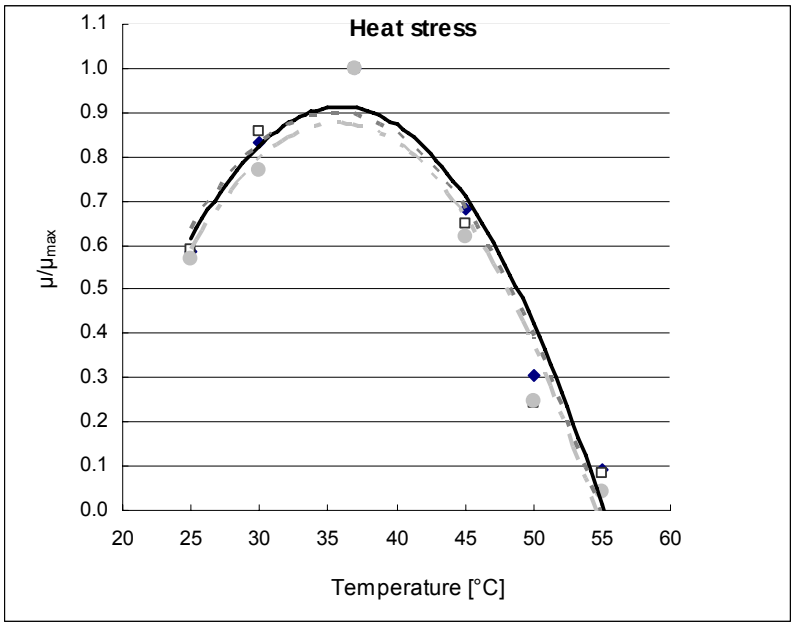

Figure 12. The change in growth rates for *Enterococcus faecalis* TMW 2.63 (●), TMW 2.629 (♦), and OG1RF (□) in response to growth in BHI broth at various temperatures (heat). Optimal growth was in BHI broth at 37°C.

3.1.4.2 Nitrite stress

Growth rates in response to nitrite stress were not determined. Instead, a single concentration of 200 ppm was used.

3.1.4.3 High hydrostatic pressure (HHP) stress

Enterococcus faecalis TMW 2.63, 2.629 and OG1RF were monitored for changes in growth during pressurization in BHI broth at intensities of 50, 80 and 100 MPa for a period of 8 h. Under these conditions, *E. faecalis* TMW 2.629 was slightly more piezotolerant than the other two strains, which responded similarly in growth reduction. However, the sub-lethal stress pressure chosen for future sub-lethal stress experiments was 80 MPa (Figure 13).

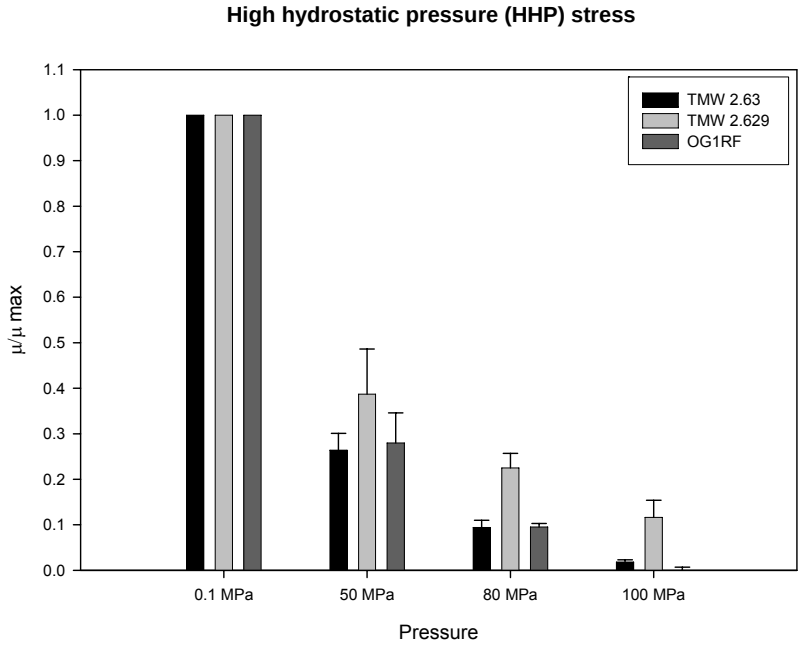

Figure 13. The change in growth rates for *Enterococcus faecalis* TMW 2.63, TMW 2.629, and OG1RF in response to growth in BHI broth under various pressures. Optimal growth was in BHI broth at 37°C held at ambient pressure (0.1 MPa).

3.1.4.4 Hyperosmotic (NaCl) stress

The growth of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF in BHI supplemented with enough NaCl to achieve concentrations of 2.0, 3.5, 4.5, 6.0, and 6.5% was determined over an 8 h period. All three strains responded similarly to the different salt stress intensities. In accordance with the definition of sub-lethal stress discussed in section 2.1.6.2, the NaCl% in BHI broth needed to reduce the growth rate of all *E. faecalis* strains was 6.8% (Figure 14).

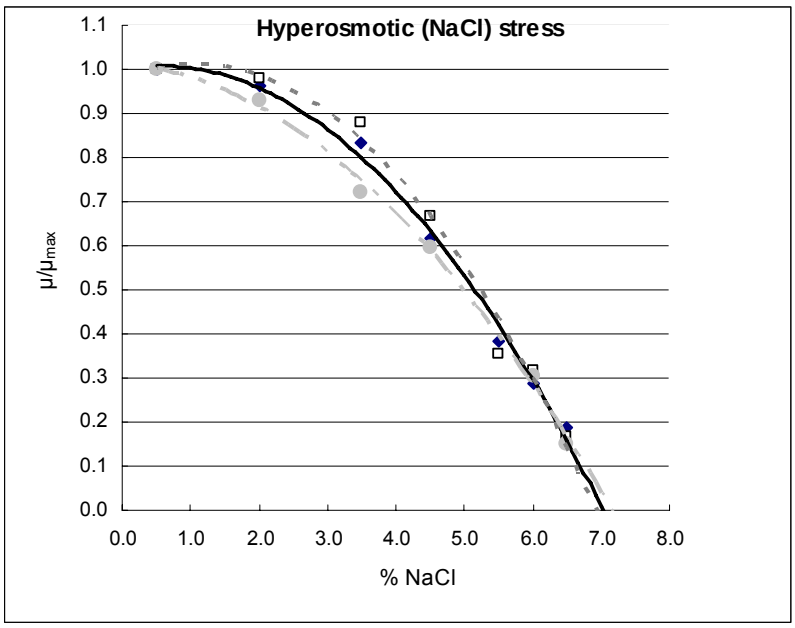

Figure 14. The change in growth rates for *Enterococcus faecalis* TMW 2.63 (●), TMW 2.629 (♦), and OG1RF (□) in response to growth in BHI broth supplemented with various NaCl%. Optimal growth was in BHI broth (0.5% NaCl) at 37°C.

3.1.4.5 Acid (HCl) stress

The growth of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF in response to acid stress was determined in BHI broth supplemented with enough HCl to adjust the pH of the growth medium to values of 3.9, 4.4, 4.9, 5.9, and 6.9 over an 8 h period. Again, all three strains responded similarly to the different degrees of acid stress, and the pH required to reduce growth to 10% of the maximum growth rate was 4.1 (Figure 15).

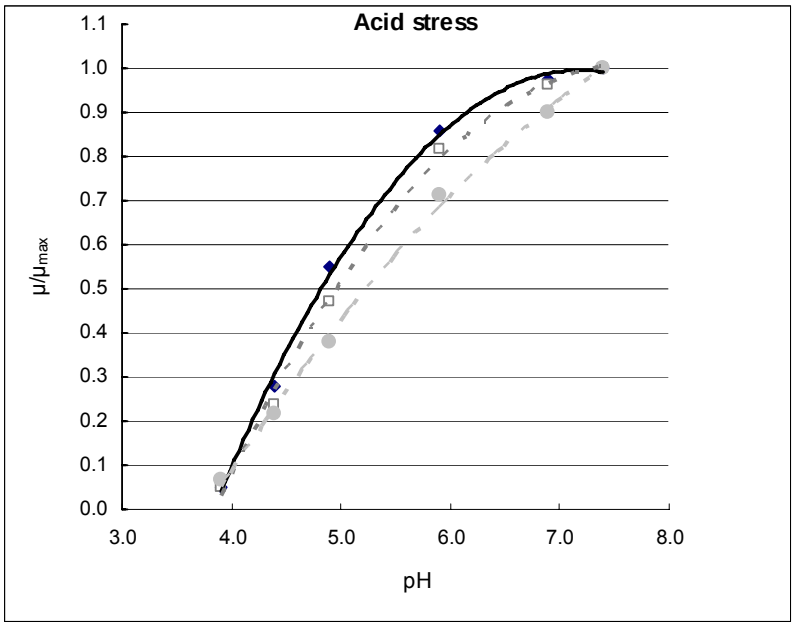

Figure 15. The change in growth rates for *Enterococcus faecalis* TMW 2.63 (●), TMW 2.629 (♦), and OG1RF (□) in response to growth in BHI broth adjusted to various acid pHs with HCl. Optimal growth was in BHI broth (pH 7.4) at 37°C.

3.1.4.6 Bile salts stress

The growth of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF in BHI broth supplemented with different amounts of bile salts, including 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.16 and 0.2% (w/v), was investigated over an 8 h period. Under these conditions, *E. faecalis* TMW 2.629 was slightly more resistant to bile salts stress than the other two strains, which responded similarly. However, a value of 0.08 % bile salts (w/v) was chosen to elicit a sub-lethal stress response according to the definition in section 2.1.6.2 (Figure 16).

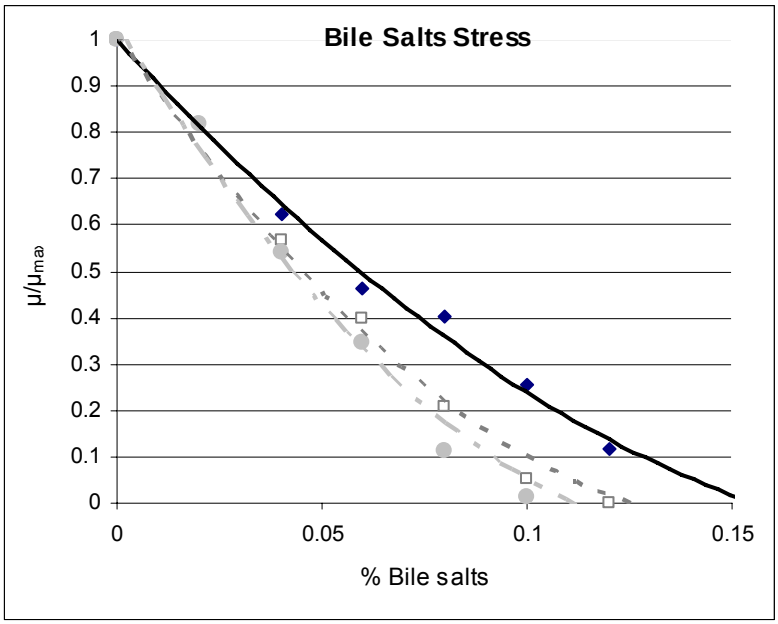

Figure 16. The change in growth rates for *Enterococcus faecalis* TMW 2.63 (●), TMW 2.629 (♦), and OG1RF (□) in response to growth in BHI broth with various concentrations of bile salts. Optimal growth was in BHI broth at 37°C with no bile salts.

3.1.5 Cross resistance between different stresses

The tolerance of *Enterococcus faecalis* TMW 2.63 cells pre-adapted to the sub-lethal stresses described above, to subsequent challenge with lethal levels of these stresses was determined. For this *E. faecalis* strain, a significant level of tolerance to subsequent lethal challenge with the same stress was observed (homologous tolerance), ranging from a factor of 312 to as high as 2765 (Table 4). However, cross-protection between different stresses varied. Bi-directional cross resistance between pH and salt stresses was observed, in which adaptation to a sub-lethal pH stress significantly increased tolerance to a lethal level of salt stress, and vice versa. Similar tri-directional cross-protection was observed among bile salts, salt, and temperature stresses at a much higher level. On the other hand, some cross-protections that worked in one direction did

not necessarily work in the opposite direction. While pre-adaptation to sub-lethal HHP stress increased tolerance to a lethal bile salts stress by a factor of 62, pre-adaptation to a sub-lethal concentration of bile salts did not provide any cross-protection against lethal HHP stress (tolerance factor of 1.1). Similar uni-directional cross-protection was observed for salt and HHP stresses. Pre-adaptation to sub-lethal salt stress increased tolerance to lethal HHP stress by a factor of 30, while pre-adaptation to sub-lethal HHP stress failed to induce tolerance to a lethal salt challenge. It is interesting to note that exposure to sub-lethal temperature, HHP and bile salts stresses all sensitize *E. faecalis* TMW 2.63 to subsequent lethal HHP and pH stresses, respectively.

Table 4. Results from the cross resistance experiments with *Enterococcus faecalis* TMW 2.63. Exponential phase cells were allowed to adapt to the pre-determined sub-lethal stresses for 1 h prior to challenge with lethal levels of each stress for 15 min. The levels of tolerance acquired to each subsequent stress were calculated by comparing the percentage of viable pretreated cells to the percentage of viable nonadapted cells. Values > 1 indicate that cross-tolerance was gained by pre-exposure to the sub-lethal stress, while values = 1 indicated that no cross-tolerance was gained. Values < 1 indicate that the first stress sensitized *E. faecalis* to the secondary stress. Homologous challenge results are highlighted in grey.

		Challenge (lethal stress)					
		Temperature (60°C)	Nitrite (0.2%)	High pressure (300 MPa)	Salt (22%)	pH (1.9)	Bile salts (0.3%)
Adaptation (sub-lethal stress)	Temperature (51°C)	2765	18.5	0.8	88	1.5	402
	Nitrite (200 ppm)	1.2	312	1.4	18	1.9	16.2
	High pressure (80 MPa)	37	1.0	323	1.2	0.5	62
	Salt (6.8%)	250	11	30	500	70	480
	pH (4.1)	2.5	19	48	63	808	1.4
	Bile salts (0.08%)	245	58	1.1	207	0.1	1748

3.2 Analysis of virulence gene expression

3.2.1 Influence of food preservation and processing conditions on virulence gene expression

3.2.1.1 Food environments – initial experiments

***clpP* expression.** The expression of *clpP* was different in each *Enterococcus faecalis* strain. In *E. faecalis* TMW 2.63, it was significantly up-regulated in late exponential phase cells upon exposure to 6.5% NaCl and pH5, while its expression was significantly down-regulated in stationary cells under the same conditions (Figure 17; Tables 5, 6). After exposure of both late exponential and stationary phase cells to 6.5% NaCl + pH5, *clpP* expression was equally up-regulated (3.2 and 3.3 fold) compared to the LB environment, while a significant amount of down-regulation compared to the LB environment was observed in late exponential and stationary grown cells exposed to the MSM condition. *E. faecalis* OG1RF demonstrated a different pattern of regulation for this gene, and down-regulated its expression in late exponential phase cells exposed to both 6.5% NaCl and pH5. However, it also significantly up-regulated *clpP* expression in both late exponential and stationary phase cells in the presence of 6.5% NaCl + pH5 (3.1 and 2.4 fold) compared to the LB environment.

***clpX* expression.** The expression of *clpX* did not reflect the patterns of expression observed for *clpP*, and was different in each *E. faecalis* strain (Figure 17; Tables 5, 6). While *E. faecalis* TMW 2.63 significantly up-regulated expression of *clpX* in late exponential phase cells exposed to MSM and pH5, *E. faecalis* OG1RF demonstrated a significant up-regulation of this gene in late exponential phase cells exposed to MSM. 6.5% NaCl, and 6.5% NaCl + pH5 environments.

***gls24* expression.** In contrast to the *clp* genes, *gls24* was significantly over expressed after exposure to almost all environments for both *E. faecalis* TMW 2.63 and OG1RF (Figure 17; Tables 5, 6). Late exponential phase cells from TMW 2.63 up-regulated *gls24* expression to a greater extent than stationary phase cells compared to their counterparts in LB after exposure to MSM, 6.5% NaCl, pH5, and 6.5% salt + pH5. After exposure to each respective supernatant, cells from both growth phases of TMW 2.63 exhibited equal up-regulation of *gls24*. On the other hand, *E. faecalis* OG1RF expressed *gls24* at a higher level in stationary grown cells compared to late exponential phase cells in both MSM and pH5 environments. A significant level of down-regulation of this gene was observed only in late exponential phase cells in the respective supernatant environment (–22.1). *gls24* expression patterns observed in 6.5% NaCl and 6.5% NaCl + pH5 remained similar to those described for *E. faecalis* TMW 2.63.

***agg* expression.** Relative levels of *agg* expression in *E. faecalis* TMW 2.63 were determined for all environments and growth phases compared to those in LB. Significant levels of down-regulation (between –2.2 and –9.6) were only observed for both late exponential and stationary phase cells exposed to MSM and 6.5% NaCl + pH5 environments (Figure 17; Table 5). Late exponential phase cells in 6.5% NaCl were also significantly down-regulated (-4.6), while stationary phase cells exposed to pH5 were similarly down-regulated (-2.7). Expression of *agg* was significantly up-regulated for both late exponential and stationary phase cells exposed to each respective supernatant (2.3 and 10.2 fold greater than LB, respectively).

***efaA* expression**. The level of *efaA* expression in all environments compared to LB was similar for both strains of *E. faecalis* (Figure 17; Tables 5, 6). While this gene was

generally down-regulated in all environments for both growth phases, after exposure to the MSM and 6.5% NaCl + pH5 environments, *efaA* was up-regulated in stationary phase cells for both *E. faecalis* strains.

***gelE* expression.** The level of *gelE* expression for both *E. faecalis* TMW 2.63 and OG1RF was determined for each of the different environments (respective supernatant, MSM, 6.5% NaCl, pH5, and 6.5% NaCl + pH5) relative to LB for both late exponential and stationary phase cells (Figure 17; Tables 5, 6). A significant increase in expression was observed for late exponential phase cells from strain TMW 2.63 after exposure to the pH5 environment (2.0 times higher) compared to cells from the same growth phase exposed to LB. Although *gelE* was down-regulated in all other environments in comparison to LB for both strains, a greater significant down-regulation was observed for *E. faecalis* OG1RF, particularly for exponential phase cells exposed to 6.5% NaCl and pH5.

***cylB* expression**. The level of *cylB* expression was only evaluated for *E. faecalis* TMW 2.63, and was significantly up-regulated in late exponential phase cells exposed to all environments, except the respective supernatants, compared to the level of expressionobserved for late exponential phase cells exposed to LB (Figure 17; Table 5). In contrast, stationary phase cells significantly down-regulated *cylB* expression in all environments compared to similarly grown cells in LB.

***cylL*$_L$ expression.** The expression of *cylL*$_L$ was also only evaluated in *E. faecalis* TMW 2.63, and demonstrated similar trends in expression as those described for *cylB*, although to a lesser extent (Figure 17; Table 5). While the levels of *cylB* up-regulation

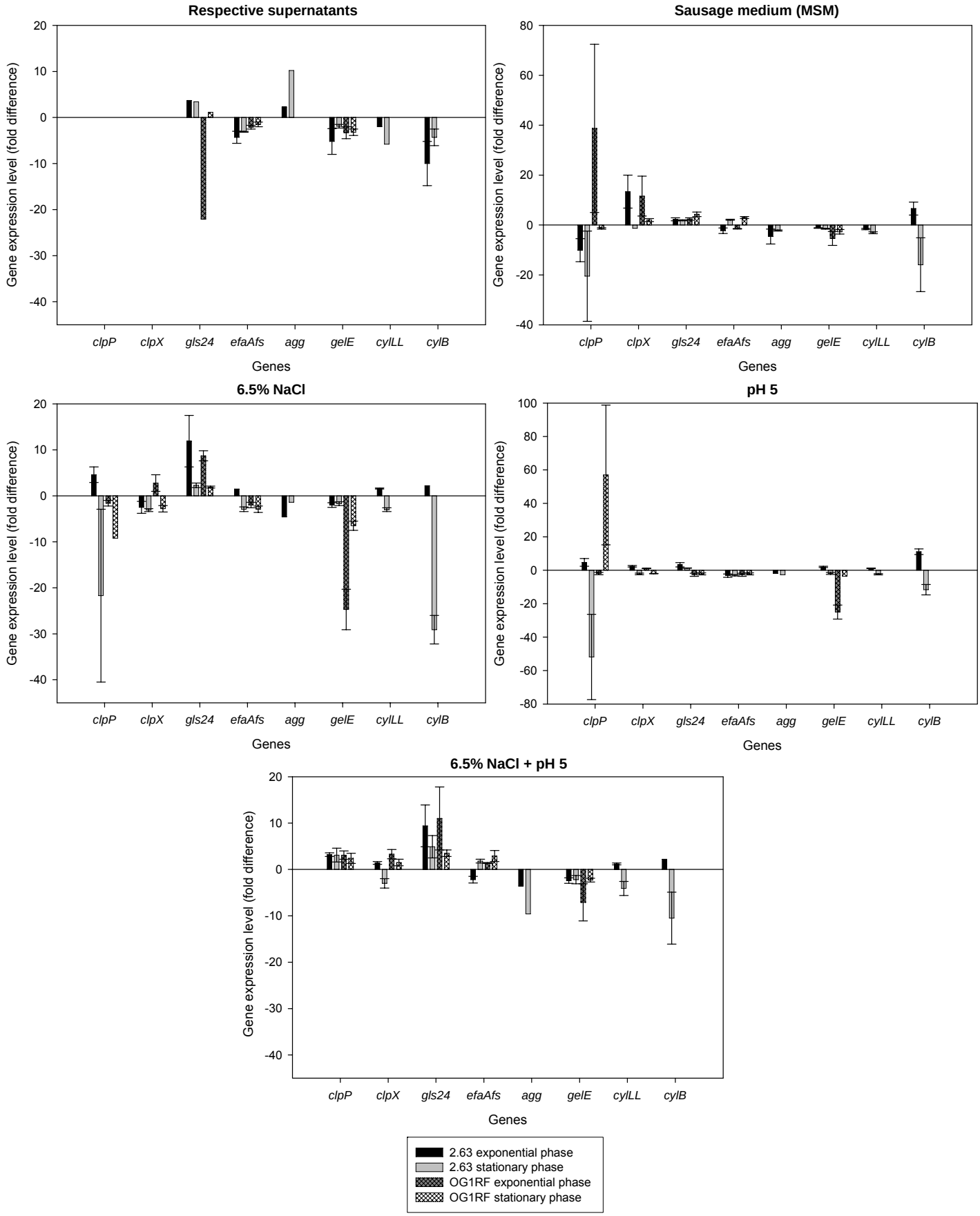

Figure 17. *Enterococcus faecalis* stress and virulence gene expression levels in response to environments encountered in food. These initial experiments examined the impact of growth phase in the *E. faecalis* strains TMW 2.63 and OG1RF.

in late exponential phase cells ranged from 2.2 to 11.1 times that observed in similarly grown cells in LB, $cylL_L$ up-regulation in late exponential phase cells only ranged from 1.2 to 1.6 times that observed after exposure to LB. Similar trends were observed for down-regulation.

3.2.1.2 Virulence gene expression patterns in response to sub-lethal stresses encountered in the food environment

***dnaK* and *groEL*.** Both of these genes were significantly up-regulated by all three *Enterococcus faecalis* strains in response to all sub-lethal stresses. Heat stress induced the transcription of these two genes to the highest levels (10-fold up-regulation of *dnaK*; 23-fold up-regulation of *groEL*), while more strain related variability in the up-regulation of these genes was observed in response to nitrite and HHP stresses (Figure 18; Table 7). The hyperosmotic and acid stresses induced similar levels of up-regulation for these two genes, with *dnaK* values between 2.2 and 2.9 and *groEL* values between 4.3 and 4.8.

***ctsR* and *clpPBCEX*.** All three *E. faecalis* strains significantly up-regulated all of these genes in response to the different sub-lethal stresses. As before, the most up-regulation in gene transcription was observed in response to heat stress. *clpX* transcription was the most highly up-regulated, while *clpB* demonstrated the most variation in expression between stresses and was only up-regulated (by factors between 2.3 and 4.5) in response to heat, nitrite and HHP stresses (Figure 18; Tables 7, 8) .

***gls24*.** The expression of this gene was not significant for any of the stress treatments, with the exception of sub-lethal nitrite stress (Figure 18; Table 7). Both *E. faecalis*

TMW 2.629 and OG1RF significantly up-regulated the transcription of this gene by factors of 3.2 and 3.6, respectively, in response to nitrite stress.

efaA. All three *E. faecalis* strains (TMW 2.63, 2.629, OG1RF) significantly up-regulated *efaA* transcription in response to sub-lethal heat, nitrite, hyperosmotic and acid stresses (Figure 18; Table 7). Induction factors ranged between 2.0 and 3.6 for all strains in response to the heat, nitrite and acid stresses. Hyperosmotic stress induced slightly more *efaA* transcriptional up-regulation, with values ranging from 5.7 to 3.8 to 4.2 for *E. faecalis* TMW 2.63, 2.629 and OG1RF, respectively.

ace. Transcription of this gene was also significantly induced by all three *E. faecalis* strains in response to all sub-lethal stresses, and the patterns for the levels of up-regulation appeared to parallel those observed for *clpX* (Figure 18; Tables 7, 8). The highest levels of *ace* transcription were observed in response to sub-lethal heat stress (between 10.4 and 13.7 fold up-regulation compared to unstressed cells), while the lowest levels of transcription were observed in response to sub-lethal acid stress (between 1.8 and 3.7 fold up-regulation compared to unstressed cells).

fsrB, gelE* and *sprE. Only *E. faecalis* strains TMW 2.629 and OG1RF possessed all three of these genes, and *fsrB* transcription was significantly up-regulated by both strains in response to all sub-lethal stresses (Figure 18; Table 7). On the other hand, *gelE* transcription was only significantly up-regulated by both *E. faecalis* strains in response to hyperosmotic and acid stresses, with values ranging from 2.3 to 4.5. For heat and nitrite stresses, only *E. faecalis* TMW 2.629 significantly up-regulated *gelE* transcription by 2.1 and 4.0 fold, respectively. HHP stress did not induce *gelE*

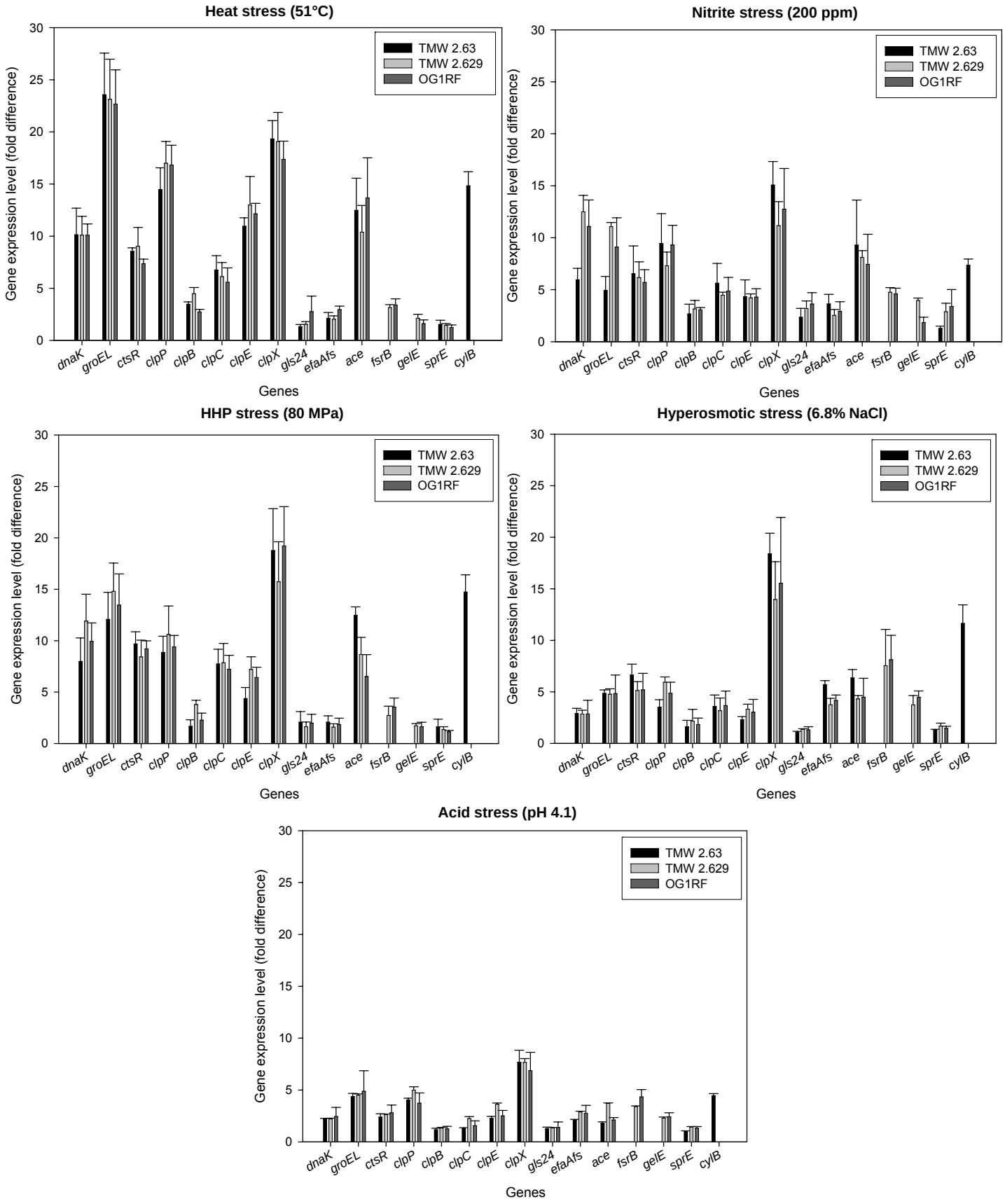

Figure 18. Stress and virulence gene expression levels of *Enterococcus faecalis* TMW 2.63, 2.629 and OG1RF in response to sub-lethal stresses encountered in the food environment and during food processing. See Appendix Table 8 for Northern (dot) blot results.

transcription. The transcription of *sprE* was only significantly induced by *E. faecalis* TMW 2.629 in response to nitrite stress (2.9 fold).

cylB. Finally, all sub-lethal stresses (heat, nitrite, HHP, hyperosmotic, acid) significantly induced the transcription of *cylB* by *E. faecalis* TMW 2.63 (Figure 18; Tables 7, 8). The highest levels were observed in response to heat and HHP stresses (14.8 and 14.7 fold up-regulation compared to unstressed conditions), and the lowest level of induction was in response to acid stress (4.5 fold induction).

3.2.2 Physiological conditions associated with the host and gut transit

3.2.2.1 Media environments used to simulate physiological conditions

***dnaK* and *groEL*.** *Enterococcus faecalis* TMW 2.63 and OG1RF significantly up-regulated the transcription of these two genes in response to bile salts stress (Figure 19; Table 7). On the other hand, *E. faecalis* TMW 2.629 only significantly up-regulated the transcription of *dnaK* by a factor of 12.9. In contrast to results obtained in response to the other sub-lethal stresses, a high degree of variation in *dnaK* and *groEL* expression levels between strains of *E. faecalis* was observed in response to bile salt stress.

***ctsR* and *clpPBCEX*.** Transcription of these genes was also up-regulated in response to sub-lethal bile salts stress and variable according to *E. faecalis* strain (Figure 19; Tables 5, 6, 7). *clpX* displayed the highest level of induction, with values ranging between 10.5 and 15.9 fold induction compared to unstressed cells.

***gls24*.** The transcription of *gls24* displayed environmental, strain, and growth phase dependent differences in regulation (Figure 19; Tables 5, 6, 7). Sub-lethal bile salts

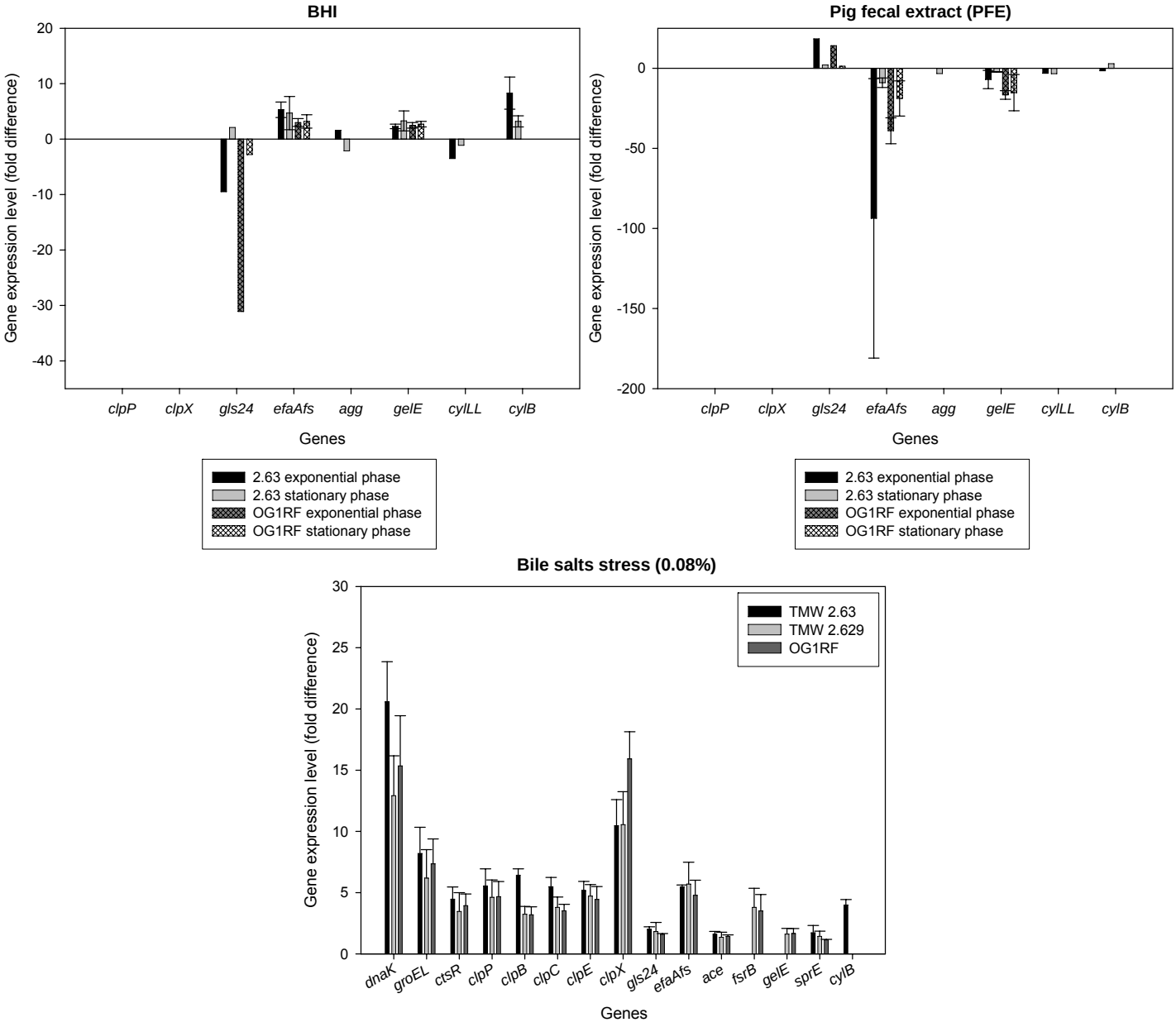

Figure 19. *Enterococcus faecalis* stress and virulence gene expression levels in response to environments encountered in the host and during gut transit.

stress only significantly induced its expression in *E. faecalis* TMW 2.63. On the other hand, in response to the BHI environment, only stationary phase *E. faecalis* TMW 2.63 cells significantly up-regulated this gene (2.1 fold). Both *E. faecalis* TMW 2.63 and OG1RF expressed *gls24* at a significantly higher level in exponential phase cells (18.4 and 14.1 fold, respectively) in the PFE environment compared to stationary phase cells (2.1 and 1.3 fold, respectively) in the same environment.

efaA. As before, the transcription of *efaA* was significantly up-regulated by all *E. faecalis* strains in response to both sub-lethal bile salts stress and the BHI environmental condition (Figure 19; Tables 5, 6, 7). Expression levels of *efaA* were not significantly different between exponential and stationary phase cells.

fsrB, gelE* and *sprE. These genes were not significantly induced by bile salt stress. However, *gelE* transcription was significantly up-regulated by both *E. faecalis* TMW 2.63 and OG1RF cells in both growth phases in the BHI environment by factors between 2.3 and 3.3 fold compared to the basic LB medium environment (Figure 19; Tables 5, 6, 7).

cylB. Finally, the transcription of *cylB* was significantly up-regulated by *E. faecalis* TMW 2.63 in response to sub-lethal bile salt stress (4.0 fold compared to unstressed conditions). Both exponential and stationary phase cells from the same *E. faecalis* strain also up-regulated this gene by factors of 8.3 and 3.2, respectively, in the BHI environment (Figure 19; Tables 5, 7).

3.2.2.2 Intestinal epithelial cells (IEC): an *in vitro* model of *Enterococcus faecalis* infection

3.2.2.2.1 Determination of bacterial cell counts

For the initial experiments with only Mode-K cells, bacterial cell counts ranged between 8.2 and 8.5 log CFU/ml at sampling time. No significant differences between control and experimental bacterial counts at sampling time were observed, with the greatest difference between the two groups equal to 0.1 log CFU/ml (Table 9). Although a similar analysis was not conducted for the experiments with all epithelial

cell lines, bacterial cell counts did not significantly differ between the inoculum used to stimulate HT-29 cells and that used to stimulate Mode-K cells, with the largest difference between the two groups equal to 0.2 log CFU/ml (Table 10).

3.2.2.2.2 Virulence gene expression patterns in response to IECs

3.2.2.2.2.1 Initial experiments with Mode-K IECs

Initial experiments with *Enterococcus faecalis* strains OG1RF, TMW 2.63 and TMW 2.629 and the mouse intestinal epithelial cell line, Mode-K were performed to characterize *E. faecalis* virulence gene expression upon contact with the host intestinal epithelium. The mouse intestinal epithelial cells were used as a comparison for experiments conducted in a mouse model (section 3.2.2.3).

While the general stress gene, *gls24*, was only slightly up-regulated by all *E. faecalis* strains upon contact with Mode-K cells, transcription of the surface protein EfaA was significantly up-regulated by all strains within the first 30 minutes of contact (Figure 20; Table 9). Only the *E. faecalis* food strain, TMW 2.629, significantly up-regulated transcription of *gelE*, and the small subunit of the excreted protease cytolysin, $cylL_L$, was not expressed under these conditions.

3.2.2.2.2.2 Follow-up experiments with HT-29 P, HT-29 MTX and Mode-K IECs

General significant gene expression levels following monoassociation with IECs. Due to possible inter-species influences on *Enterococcus faecalis* virulence gene expression, the influence of human colon carcinoma cell lines, HT-29 P and HT-29 MTX, on *E. faecalis* virulence gene regulation was investigated and compared to the response observed for Mode-K cells. The two isogenic mutants of *E. faecalis* OG1RF were also included in these investigations to determine if the absence of the *fsrB* and

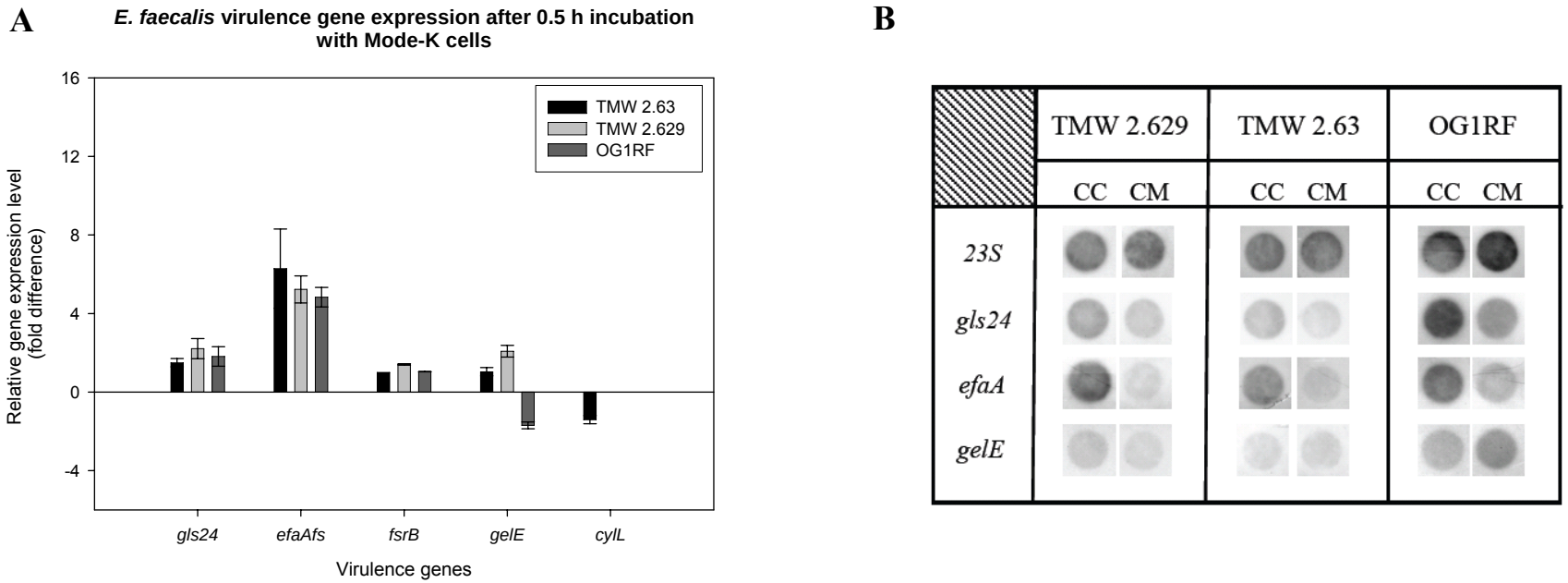

Figure 20. *Enterococcus faecalis* virulence gene expression levels in response to the presence of Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology (A) and by Northern (dot) blotting (B), in which *23S* was used as a housekeeping gene.

Table 9. Virulence gene expression levels for the different *Enterococcus faecalis* strains after 30 minutes of monoassociation with Mode-K cells. Numbers reflect the fold difference in expression of the different genes in the presence of cell culture (CC) relative to their expression in the presence of only cell culture medium (CM). Significant expression levels ($p<0.05$) are written in bold. The *E. faecalis* cell counts at sampling time were also determined to check that the calculated differences in gene expression were not attributed to significant variations in cell concentration.

Enterococcus faecalis strains	Virulence genes					Cell counts at sampling time (log CFU/ml)	
	gls24	*efaA*	*fsrB*	*gelE*	*cylL*$_L$	CC	CM
TMW 2.63	1.5 ±0.2	6.3 ±2.0	1.0 ±0	1.0 ±0.2	-1.4 ±0.2	8.2 ±0.2	8.3 ±0.1
TMW 2.629	2.2 ±0.5	**5.2** ±0.7	1.4 ±0	**2.1** ±0.3		8.4 ±0.1	8.5 ±0
OG1RF	1.8 ±0.5	**4.8** ±0.5	1.0 ±0	-1.7 ±0.2		8.2 ±0	8.3 ±0

gelE genes influenced virulence gene expression under the study conditions and could be compared to observations made using the *in vivo* mouse model.

Genes reported to play a role in *E. faecalis* virulence were evaluated for their regulation in the presence of two human colon carcinoma cell lines (HT-29 P, HT-29 MTX), a mouse intestinal cell line (Mode-K), and their respective cell culture media (CCM). Changes in gene expression due to the presence of intestinal cells were determined in exponential phase *E. faecalis* isolates by comparing mRNA abundances for the different genes under these conditions relative to CCM. Significant differences in gene expression ($p<0.05$) were determined by comparing values greater than 1 to a threshold of 1, and those less than 0.5 to a threshold of 0.5 using the Student's t test.

One of the first virulence genes analyzed for differential gene expression in response to intestinal cells was the general stress gene, *gls24*. While only minor changes were observed in *E. faecalis* TMW 2.63, 2.629, and OG1RF, the two OG1RF mutant strains significantly down-regulated this gene in the presence of HT-29 P and Mode-K cells (Figures 21–25; Table 10).

The next group of virulence genes evaluated for significant changes in expression due to the presence of intestinal cells, encode surface proteins. Minor changes in expression were observed for *esp* (Table 10), while *E. faecalis* TMW 2.629 and TX5266 both significantly down-regulated *ace* in the presence of Mode-K cells (Figures 22, 25; Table 10). This trend was also observed for the other *E. faecalis* isolates, although not at a significant level. All *E. faecalis* isolates significantly up-regulated *efaA* in the presence of all intestinal cell types with the exception of TMW 2.63, which only up-regulated *efaA* in the presence of Mode-K cells (Figures 21–25; Table 10).

Differences in expression were also investigated for virulence genes involved in the regulation and excretion of proteases. *E. faecalis* TMW 2.63 was the only isolate with both the genotype and phenotype for the production of cytolysin, and did not significantly change expression of $cylL_L$ (the large subunit of cytolysin) or *cylB* (an ATP-binding cassette that transports cytolysin across the membrane) in the presence of any of the intestinal cells (Figure 21; Table 10). On the other hand, *E. faecalis* TMW 2.629 and OG1RF, which possess all genes required for the expression of *gelE* (a metalloprotease) and *sprE* (a serine protease), both significantly up-regulated *fsrB* (the signal peptide for transcription of *gelE* and *sprE*) and *gelE* in the presence of intestinal epithelial cells (Figures 22, 23; Table 10). Although *sprE* was also up-regulated under these conditions by both *E. faecalis* isolates, it was only significant for OG1RF in the presence of HT-29 MTX cells ($p<0.05$). The two knockout mutants of *E. faecalis* OG1RF had expression patterns consistent with their deletions. *E. faecalis* TX5264, which contains a deletion knockout of *gelE*, significantly up-regulated *fsrB* and *sprE* in the presence of intestinal epithelial cells, while TX5266, which contains a deletion knockout of *fsrB,* did not express any of the genes under these conditions (Figures 24, 25; Table 10).

Influence of cell type on *in vitro* virulence gene expression. The influence of intestinal epithelial cell type on *E. faecalis* virulence gene expression was investigated using undifferentiated (HT-29 P) and differentiated (HT-29 MTX) human colon carcinoma cell lines and a mouse intestinal epithelial cell line (Mode-K). Relative gene expression values were calculated using a comparison between quantified mRNA for the different virulence genes from samples taken after incubation with the different epithelial cell lines and samples taken after incubation with only CCM. Significant

Table 10. *Enterococcus faecalis* virulence gene expression levels in response to intestinal epithelial cells (IECs). Cell lines included two human colon carcinoma cell lines, HT-29 P (undifferentiated) and HT-29 MTX (differentiated), and a mouse small intestinal cell line, Mode-K. Expression levels were determined after 30 min monoassociation with each *E. faecalis* strain. Statistically significant values ($p<0.05$) are in bold and highly up-regulated values are highlighted in grey. See Appendix for Northern (dot) blot results.

Enterococcus faecalis isolate	Cell culture type	Virulence genes									Cell counts at sampling (log CFU/ml)
		gls24	*efaA*	*esp*	*ace*	*fsrB*	*gelE*	*sprE*	*cylL$_L$*	*cylB*	
TMW 2.63 (clinical)	HT-29 MTX	-1.5 ±0	-1.3 ±0		**1.5** ±0.1		-1.6 ±0.1	-1.1 ±0	1.0 ±0.2	-1.8 ±0.1	7.9
	HT-29 P	-1.4 ±0.1	-1.3 ±0.1		2.5 ±0.7		-1.1 ±0.1	1.6 ±0.6	1.4 ±0.1	-1.2 ±0.2	
	Mode-K	1.3 ±0.2	3.4 ±1.7		-1.8 ±0.1		-1.3 ±0.2	1.4 ±1.1	1.1 ±0.1	-1.4 ±0.5	7.9
TMW 2.629 (food)	HT-29 MTX	1.3 ±0.2	**3.1** ±0.2	-1.2 ±0.1	-1.8 ±0.2	**3.3** ±0.3	**2.3** ±0.4	1.7 ±0.3			8.5
	HT-29 P	2.0 ±0.8	4.2 ±1.3	1.9 ±0.7	-1.3 ±0.3	**6.8** ±0.5	3.8 ±1.5	-1.4 ±0.1			
	Mode-K	-2.1 ±0.1	**3.4** ±0.8	-1.4 ±0.1	**-5.8** ±0	**2.7** ±0.3	**2.0** ±0.2	3.0 ±0.8			8.4
OG1RF (well studied, clinical)	HT-29 MTX	-1.3 ±0.2	**2.4** ±0.5		1.1 ±0.4	1.8 ±0.3	**1.8** ±0.2	**4.0** ±1.5			8.3
	HT-29 P	-2.5 ±0.2	**2.6** ±0.6		1.1 ±0.3	1.8 ±0.6	**1.8** ±0.3	2.3 ±0.8			
	Mode-K	1.0 ±0.3	**3.3** ±0.6		-1.1 ±0.4	1.3 ±0.1	**2.8** ±0.7	6.1 ±2.6			8.4
TX5264 (OG1RFΔ*gelE*)	HT-29 MTX	-3.2 ±0.1	**2.0** ±0.3		-1.8 ±0	**2.4** ±0.2	0	**2.4** ±0.2			8.3
	HT-29 P	**-4.2** ±0.1	1.8 ±0.6		-1.6 ±0.2	1.9 ±1.2	0	2.6 ±1.2			
	Mode-K	**-2.9** ±0.1	**1.8** ±0.2		-2.6 ±0.1	1.7 ±0.2	0	**6.2** ±1.9			8.3
TX5266 (OG1RFΔ*fsrB*)	HT-29 MTX	-1.8 ±0.3	1.8 ±0.6		-1.9 ±0.2	0	-1.0 ±0.3	0			8.3
	HT-29 P	**-3.5** ±0	**1.6** ±0.1		-2.2 ±0.1	0	-1.1 ±0.1	0			
	Mode-K	-2.4 ±0.1	2.7 ±1.0		**-4.9** ±0.1	0	-1.0 ±0.3	0			8.0

differences in expression in response to the different cell lines were calculated using comparisons between the cell lines for each virulence gene using the Student's t test ($p<0.05$).

Differences in expression for *gls24*, the general stress gene, were observed between the cell types. However, the only significant difference ($p<0.05$) in gene expression was between the HT-29 MTX and Mode-K cells (Table 10), where *gls24* was significantly lower in the presence of Mode-K cells.

Evaluation of differences in virulence gene expression in response to the different cell types among the surface proteins revealed significant variations in *ace* expression. *E. faecalis* TMW 2.63, TX5264 and TX5266 all had significantly lower expression of *ace* in the presence of Mode-K cells in comparison to HT-29 MTX or HT-29 P (Table 10). While this effect was not significant for *E. faecalis* OG1RF and TMW 2.629, a similar trend was observed.

Genes involved in the regulation and excretion of proteases were also evaluated for significant differences in expression due to intestinal epithelial cell type. *E. faecalis* TMW 2.629 significantly up-regulates *fsrB* in the presence of HT-29 P cells compared to both HT-29 MTX and Mode-K cells (Table 10), although similar significant differences for *gelE* and *sprE* were not observed. Significant differences in expression in response to the different cell types were not observed for the other virulence genes.

3.2.2.3 Mouse model: an *in vivo* model of *Enterococcus faecalis* infection

3.2.2.3.1 Exploratory mouse study – what genes are expressed?

Preliminary experiments conducted to determine if differences in *Enterococcus faecalis* gene expression in a mouse model could be observed were conducted based upon an expressed or not expressed basis. The mouse experiments were carried out by Micha

Hoffmann and Dirk Haller (Biofunktionalität der Lebensmittel, Technische Universität München), while the collected intestinal contents (containing the bacteria) from these studies were analyzed in this work. *E. faecalis* OG1RF expressed both *efaA* and the pheromone *ccf* in both IL-$10^{-/-}$ and WT mice at all sampling times. On the other hand, *E. faecalis* OG1RF expressed *gelE* after 98 days of monoassociation with IL-$10^{-/-}$ mice, while *gelE* expression was notably absent in WT mice for all time points (Table 11). This observation correlated with the development of colonic inflammation that was absent at earlier sampling times, and significantly higher in IL-$10^{-/-}$ mice compared to WT mice (Dirk Haller, personal communication, unpublished data), which prompted further investigation.

Table 11. Exploratory analysis of *Enterococcus faecalis* virulence gene expression in a mouse model during the course of infection development (time dependent evaluation). A '+' indicates expression while a '-' indicates no expression.

Bacterium	Mouse type	Sampling time	Virulence gene expression						
			efaA	*gelE*	*agg*	*esp*	*cpd*	*cob*	*ccf*
OG1RF	IL-$10^{-/-}$ mice	Day 7	+	-	-	-	-	-	+
		Day 14	+	-	-	-	-	-	+
		Day 98	+	+	-	-	+	-	+
OG1RF	WT mice	Day 7	+	-	-	-	+	-	+
		Day 14	+	-	-	-	-	-	+
		Day 98	+	-	-	-	-	-	+

3.2.2.3.2 Follow-up study: Mouse histology

The histology analysis was conducted by Micha Hoffmann and Dirk Haller and was presented by Hoffmann et al. (Hoffmann, 2007) at the 13th International Congress of Mucosal Immunology, 2007. These results are presented here to portray the conditions in the mouse at the time *Enterococcus faecalis* samples were taken along with the intestinal contents (see section 2.2.5.3.2). Briefly, all tissue samples taken from WT

mice monoassociated with all *E. faecalis* strains had little inflammation, with histology scores consistently less than 1 (little or no inflammation). On the other hand, for all tissue samples from IL-10$^{-/-}$ mice, *E. faecalis* OG1RF was consistently associated with a significantly higher amount of inflammation compared to the *E. faecalis* mutants TX5264 and TX5266, with the highest amount occurring in the distal colon (Dirk Haller, personal communication, unpublished data;(Hoffmann, 2007)).

3.2.2.3.3 Follow-up study: Determination of intestinal bacterial counts in WT and IL-10$^{-/-}$ mice

The number of bacteria in combined colon and cecum samples taken from each group of mice was determined. *Enterococcus faecalis* concentrations (log CFU/g fecal matter) were similar for all mice and all *E. faecalis* isolates, ranging from 8.0 to 8.8 log CFU/g fecal matter (Table 12).

3.2.2.3.4 Follow-up study: Bacteria verification from colonized WT and IL-10$^{-/-}$ mice

Bacteria samples isolated from the combined mouse colon and cecum contents were genotypically confirmed using PCR. The expected genotypes were confirmed (Figure

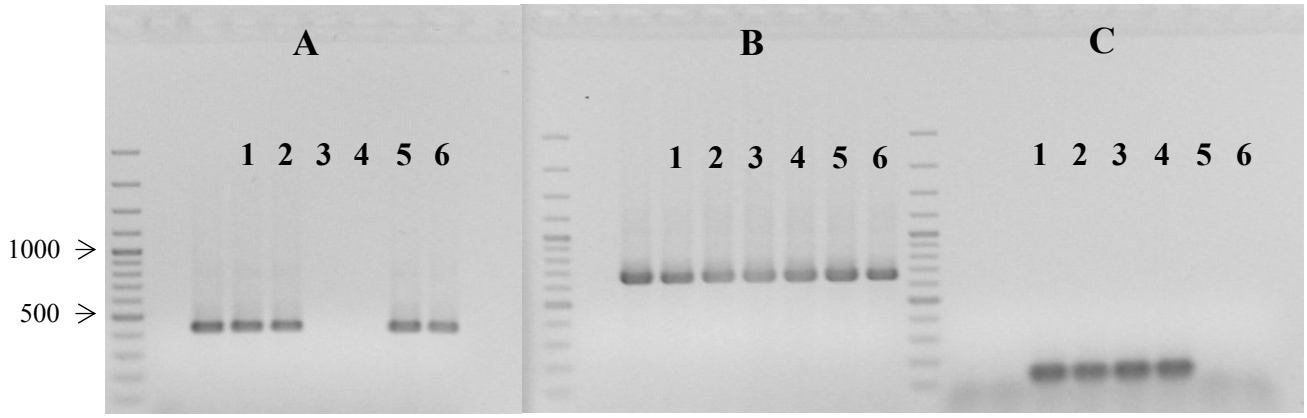

Figure 26. Bacteria verification from mouse intestinal samples. PCR results for *Enterococcus faecalis* isolated from IL-10$^{-/-}$ and WT mice for the virulence genes *gelE* (A), *sprE* (B) and *fsrB* (C). *E. faecalis* isolates were confirmed genotypically to be OG1RF (1 and 2), TX5264 (3 and 4), and TX5266 (5 and 6).

26).

3.2.2.3.5 Follow-up study: Virulence gene regulation patterns in the mouse

As in the previous section, genes reported to play a role in *Enterococcus faecalis* virulence were evaluated for the level of expression during the actual infection process in a mouse model. *E. faecalis* OG1RF and two isogenic deletion mutants for *gelE* and *fsrB* (TX5264 and TX5266, respectively) were monoassociated with IL-10$^{-/-}$ and WT mice for 15 weeks before intestinal contents from the colon and cecum were combined and *E. faecalis* RNA was extracted. The expression of the different *E. faecalis* virulence genes in IL-10$^{-/-}$ mice was determined relative to that in WT mice based on mRNA abundances for the virulence genes. Significant differences in gene expression ($p<0.05$) were determined by comparing values greater than 1 to a threshold of 1, and those less than 0.5 to a threshold of 0.5 using the Student's t test.

Differences in expression of the general stress gene, *gls24*, in IL-10$^{-/-}$ compared to WT mice were evaluated. While this gene was down-regulated in IL-10$^{-/-}$ mice (Figure 27; Table 12), these results were not significant ($p>0.05$) and the regulation of this gene did not appear to be significantly influenced by the absence of *gelE* or *fsrB*.

The second group of virulence genes analyzed for differences in expression in IL-10$^{-/-}$ mice relative to WT mice included surface proteins. *E. faecalis* TX5264 significantly up-regulated *ace* in IL-10$^{-/-}$ mice (6.2 fold higher than in WT mice), and this effect was seen to a lesser extent for *E. faecalis* OG1RF (Figure 27; Table 12). No difference in *ace* expression by *E. faecalis* TX5266 was observed between the groups of mice. On the other hand, all *E. faecalis* isolates up-regulated *efaA* after 15 weeks of monoassociation with IL-10$^{-/-}$ mice compared to WT mice. However, only *E. faecalis* OG1RF significantly up-regulated *efaA* ($p<0.05$), which correlated with the

development of severe inflammation in the colonic epithelium of IL-10$^{-/-}$ mice (see histology section 3.2.2.3.2).

Differences in the expression of virulence genes involved in the gelatinase pathway were observed in IL-10$^{-/-}$ mice compared to WT mice. Although *fsrB* was only slightly up-regulated by *E. faecalis* TX5264 (Δ*gelE*) and slightly down-regulated by *E. faecalis* OG1RF, both isolates highly up-regulated *sprE* and *gelE* expression (respectively) in IL-10$^{-/-}$ mice compared to WT mice. *E. faecalis* TX5264, which did not express *gelE*, up-regulated *sprE* by 8.7 fold, while OG1RF up-regulated *gelE* by 4.0 fold (Figure 27; Table 12). No significant changes in these genes were observed for *E. faecalis* TX5266.

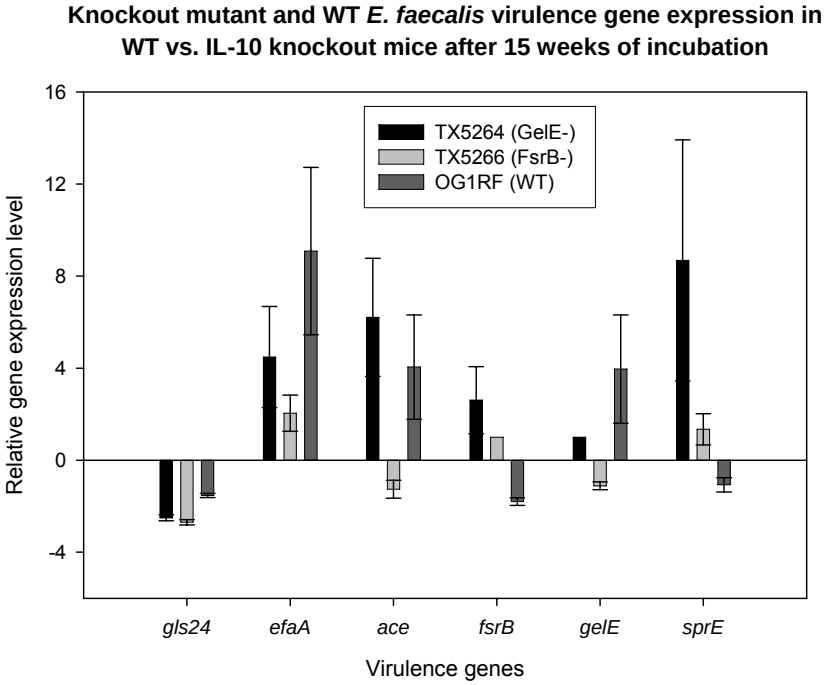

Figure 27. Virulence gene expression levels of *Enterococcus faecalis* OG1RF and two of its isogenic mutants, TX5264 *(ΔgelE*) and TX5266 (*ΔfsrB*), after 15 weeks of monoassociation with IL-10$^{-/-}$ and WT mice. mRNA quantification of gene expression was performed with RT-PCR.

Table 12. Virulence gene expression levels of *Enterococcus faecalis* after 15 weeks of monoassociation with mice determined with RT-PCR. *E. faecalis* OG1RF and two isogenic deletion mutants, TX5264 and TX5266, for *gelE* and *fsrB*, respectively, were monoassociated with IL-$10^{-/-}$ and WT mice before intestinal samples were collected and evaluated for *E. faecalis* virulence gene expression at the time of sampling. Statistically significant values ($p<0.05$) are in bold.

Enterococcus faecalis isolate	Virulence genes						Cell counts (log CFU/ml)	
	gls24	*efaA*	*ace*	*fsrB*	*gelE*	*sprE*	WT	IL-$10^{-/-}$
OG1RF	-1.5 ±0.1	**9.1** ±3.6	4.1 ±2.3	-1.8 ±0.2	4.0 ±2.4	-1.1 ±0.31	8.8 0.2	8.4 0.4
TX5264 (Δ*gelE*)	-2.5 ±0.1	4.5 ±2.2	**6.2** ±2.6	2.6 ±1.5	0	8.7 ±5.2	8.8 0.1	8.0 0.3
TX5266 (Δ*fsrB*)	-2.7 ±0.1	2.0 ±0.8	-1.3 ±0.4	0	-1.1 ±0.2	1.3 ±0.7	8.7 0.2	8.0 0.3

4 Discussion

The apparent Janus face of *Enterococcus*, which may serve both health and illness at the same time, is not at all a contradiction. Obviously, some traits known to be involved in pathogenicity are also expected to be expressed by effective probiotics and may be required when it comes to the use of bacteria in the therapy of chronic diseases. Among these traits should be factors determining pH-tolerance, ability to survive intestinal passages, adhesion, persistence, as well as influencing the intestinal flora and immune system. Some of these traits are termed virulence factors. Therefore, a closer look at the expression of virulence factors may be a good starting point to develop a rational view on bacteria used in respective applications providing at the same time information on possibilities to limit clinical infection.

4.1 *E. faecalis* strains and characterization

4.1.1 Virulence genes and phenotypes

The examination of *Enterococcus faecalis* strains for the presence of virulence factors has become a commonly suggested method to determine if a strain is "safe" for use in food applications. Since most strains possess at least one suspected virulence factor, without necessarily becoming virulent, it is impossible to predict the safety of *E. faecalis* strains used in foods or as probiotics based solely upon the presence or absence of virulence genes. Results of the initial screening of *E. faecalis* isolates from clinical samples, foods, and animals from the present study underscore the inability to predict pathogenesis based upon the presence or absence of virulence genes alone, as strains from all origins possessed different sets of between nine and fifteen virulence genes. Other studies conducted to compare the number of virulence genes found in food versus clinical isolates have had similar results (Creti *et al.*, 2004; Eaton and Gasson,

2001; Semedo *et al.*, 2003), which indicates that perhaps environmental factors, rather than the presence or absence of virulence genes, are more influential in the establishment of infection in the host.

This result is more striking if one considers that the presence of various virulence genes investigated in the present study did not necessarily correlate with the projected phenotype. While in the case of cytolysin, the only tested strain both had the *cyl* genes and expressed the phenotype, of the twelve *E. faecalis* isolates that possessed *gelE* only seven actually produced gelatinase. Studies conducted to determine the regulatory mechanisms behind gelatinase expression reported that *fsrB* is required for *gelE* transcription and translation, which is regulated in a quorum-sensing manner (Nakayama *et al.*, 2001; Qin *et al.*, 2000, 2001). This finding holds true in this case as well, as only the *E. faecalis* strains that had both *fsrB* and *gelE* expressed gelatinase. Bourgogne et al. (Bourgogne *et al.*, 2006) suggest that *fsrB* may play a greater role in the *E. faecalis* virulence process, as its absence appeared to affect a number of other genes and resulted in decreased virulence in animal models (Engelbert *et al.*, 2004; Mylonakis *et al.*, 2002; Sifri *et al.*, 2002). However, one of the strains in the current study that had *gelE* but did not produce gelatinase due to the absence of *fsrB* was a clinical isolate (i.e. infection-derived), which implies that more factors are involved in the switch to pathogenicity than the presence or absence of this prominent virulence gene or its regulator, *fsrB*. Other studies have reported similar results (conclusions)(Roberts *et al.*, 2004), which accentuates the need to focus more on how the environment encountered in foods during preservation or processing, as well as during gut transit in the host influences *E. faecalis* gene expression. Thus, it is imperative that the mechanisms (environmental conditions) which influence virulence gene expression, or the conversion to pathogenesis, be understood.

4.1.2 Environmental stresses – determination of sub-lethal stress, cross-protection and sensitization

In order to begin the investigation of how environmental conditions influence *Enterococcus faecalis* virulence gene expression, these conditions needed definition. Initially, various conditions which reflected those found both in foods and in the host were chosen, and included the excreted products from both growing and starvation stressed *E. faecalis* strains (quorum sensing), a simulated sausage medium (MSM), high salt concentration (6.5%) and low pH (5) both separately and combined, Brain Heart Infusion (BHI) medium, and a porcine fecal extract (PFE), which simulated the conditions within the digestive tract of pigs (see sections 4.2 and 4.3 for a discussion on these parameters). However, these conditions only represent distinct parameters found in foods and parts of the host. The sub-lethally stressed population of *E. faecalis* also needs consideration in regard to food safety. Therefore, sub-lethal stress conditions were defined for heat, nitrite, high pressure, NaCl, acid pH, and bile salts stress. While nitrite, salt (used to bind free water and lower the water activity in foods), and acid are added to foods to inhibit bacterial growth or eliminate bacteria altogether, heat and high pressure are mechanical food processing conditions used to accomplish this goal. The additional stress of digestion encountered in the intestinal tract of the host is represented by bile salts, which are released by the gall bladder and act to break down fats through a mechanism similar to a detergent. As previously mentioned in section 2.1.6.2, sub-lethal stress was defined as the point at which the maximum growth rate was reduced to 10% after 1 hour of the applied stress. Although there were slight variations in these values between the *E. faecalis* strains tested, they generally responded the same way to all investigated stresses (section 3.1.4) and thus, the sub-lethal stresses of 51°C, 200 ppm nitrite, 80 MPa of pressure, 6.8% NaCl, pH 4.1, and 0.08% bile salts were used to evaluate the sub-lethal stress response of *E. faecalis*.

An interesting aspect of sub-lethal stress in foods is the ability of bacteria to acquire cross-protection against or sensitization to a subsequent lethal stress. This means that the food environment and the order of environmental challenges applied during food processing and after consumption by the host may either increase the hardiness of *E. faecalis* or contribute to its demise. Evaluation of this aspect of bacterial responsiveness is an important part of food safety, and can help food processors design effective processing parameters to ensure that potential foodborne pathogens like *E. faecalis* are effectively eliminated from foods. For *E. faecalis* strain TMW 2.63, while pre-adaptation to all sub-lethal stresses provided significant protection against subsequent challenge with the same stress at lethal concentrations (homologous tolerance), tolerance to non-homologous lethal stress challenges (cross-protection) varied.

Homologous tolerance has already been observed in *E. faecalis* exposed to a number of different stresses. These include acid (pre-treatment pH 4.8, lethal stress pH 3.2), heat (pre-treatment at 50°C, lethal stress at 62°C) and bile salts (pre-treatment at 0.08%, lethal stress at 0.3%), which significantly increased the tolerance factor, or the ratio of percent viability of pre-treated cells to the percent viability of non-adapted cells, to lethal levels of each stress to 13,000, 650 and 1,700, respectively (Flahaut *et al.*, 1996c; Flahaut *et al.*, 1997; Rince *et al.*, 2003). However, the effects of salt, nitrite and high hydrostatic pressure have not yet been evaluated in *E. faecalis*. In the present study, sub-lethal pre-stress in these environments significantly increased the tolerance of *E. faecalis* TMW 2.63 to subsequent lethal homologous stress. While a detailed explanation for this phenomenon was not found in the literature, it seems obvious that adaptation to a sub-lethal level of a given stress allows the bacteria to already initiate the transcription/translation of the required specific stress proteins in preparation for a

lethal challenge of the same stress. Thus, tolerance to homologous stress is often much higher than that between different stresses.

The development of cross resistances between different stresses encompasses an interesting area of bacterial stress resistance. A number of bi- and even tri-directional cross resistances were observed between stresses, and included acid/salt as well as salt/heat/bile salt cross resistances. As with homologous stress resistance, the acquired cross-protections observed within these two groups of stresses implies that there is some overlap in the *E. faecalis* stress response to these conditions, i.e. these stress responses are closely related. Indeed, in regard to the second tri-directional group of cross resistances, (Flahaut *et al.*, 1996c) found that 18 out of 21 bile salt stress polypeptides are also induced by heat, and (Rince *et al.*, 2003) reported that 37 of 96 proteins expressed in response to salt stress are also induced by bile salts. However, the exact mechanisms that *E. faecalis* uses to deal with these stresses has not yet been thoroughly investigated.

Proteomic studies have attempted to trace a path between these cross resistances by determining if protein expression in response to one stress is required for the development of tolerance against a subsequent stress of another type. This was accomplished by the inclusion of chloramphenicol, which blocks protein synthesis, during adaptation to the first stress. Subsequent failure of the first stress to provide cross-protection against the second stress implied that some or all of the proteins expressed in response to the first stress were required for tolerance against the second stress. On the other hand, failure of blocked protein synthesis during the first stress to impart sensitivity to the second stress implied that the expression of other proteins is involved in the observed cross-protection, although the exact mechanisms for this remain undiscovered. Flahaut et al. (Flahaut *et al.*, 1997) found that the addition of

chloramphenicol during the adaptation of *E. faecalis* ATCC19433 to both 0.08% bile salts and 6.5% NaCl prevented the development of the previously observed cross-protection against a subsequent heat stress of 62°C. This implies that some or all of the proteins expressed in response to these stresses are required for the development of heat tolerance. However, another study conduced by (Flahaut *et al.*, 1996a) found that chloramphenicol-blocked protein synthesis during 6.5% salt adaptation had no effect on the tolerance development of *E. faecalis* ATCC19433 to a subsequent lethal 0.3% bile salts stress. Thus, the mechanisms behind the development of this type of cross resistance remain unclear, and perhaps involve adaptation at the transcriptional rather than the translational level.

This hypothesis may also apply to the uni-directional cross resistances observed for *E. faecalis* TMW 2.63. While sub-lethal HHP stress and salt stress provide cross-protection against subsequent lethal stresses with bile salts and HHP, respectively, the inverse of these stress applications fails to provide cross-protection. Additionally, while the sub-lethal stresses with pH and HHP increase tolerance to lethal HHP and temperature by factors of 48 and 37, respectively, the inverse of these applications actually sensitizes *E. faecalis* TMW 2.63 to the lethal stresses (both tolerance factors were < 1). As the response of *E. faecalis* to HHP and how this affects cross-protection against or sensitization to subsequent lethal stresses has not yet been investigated, one can only hypothesize about the reasons behind these results. In order to accomplish this, an understanding of the manner in which these stresses affect the bacteria as well as an analysis of the *E. faecalis* stress response at the transcriptional level were combined to present a more complete story in the following sections.

However, based upon the results of the cross-protection study conducted with *E. faecalis* TMW 2.63 a general conclusion regarding food safety can be made. The order

of applied stresses has a large impact on the survivability of *E. faecalis*. While many of the chemical and physical stresses applied in the food processing environment either increase or have no impact on the tolerance of *E. faecalis* TMW 2.63 to other stresses in this group, two different applications actually sensitized this *E. faecalis* isolate to additional stress. Sub-lethal high temperature stress increased sensitivity to a subsequent lethal HHP stress, and a sub-lethal HHP stress increased sensitivity to a subsequent lethal acid stress, a result which should be considered during the design of food processing applications. On the other hand, it is interesting to note that these various sub-lethal stresses fail to sensitize *E. faecalis* TMW 2.63 to a subsequent lethal dose of bile salts stress, which implies that, at least for this *E. faecalis* isolate, sub-lethally stressed cells can survive transit through the host gastrointestinal tract, and should therefore be eliminated in the food product prior to ingestion.

4.2 *Enterococcus faecalis* virulence gene expression in response to food preservation and processing conditions

While much attention has focused on the identification of virulence factors/genes and their impact on the pathogenicity of *Enterococcus faecalis*, these studies do not consider the numerous environmental stresses encountered by *E. faecalis* in foods and during food processing which may contribute to the switch to pathogenicity. As previously mentioned in the introduction, *E. faecalis* is a hardy organism that is able to survive harsh environmental conditions pertaining to temperature, salt (osmotic stress), and pH. Additionally, *E. faecalis* strains chosen for use in food applications as fermentation organisms or probiotics often possess traits which make them dually suited to act as both helpful and harmful organisms—helpful in foods, harmful as pathogens. Our studies of how a sub-lethally stressed *E. faecalis*

population responds to these conditions on a transcriptional level provide insight into this unexplored area of *E. faecalis* food safety.

Foods are comprised of numerous components which can both stress and protect the bacteria present in this environment. While the addition of water binding agents like salt or sugar, drying, or adjusting the pH to acidic or basic levels helps to control (sub-lethally stress) or eliminate the bacteria population within the food, other components such as fats help to protect bacteria from these potentially deleterious conditions (Smith, 2003). This creates pockets of virile or sub-lethally stressed bacteria within a food medium which are in turn, transferred to the host when the food item is eaten. As the bacterial response to stress often coincides with increased virulence (Abee and Wouters, 1999; Arnold *et al.*, 2007; Henderson *et al.*, 2006; Rowley *et al.*, 2006), the stress conditions that *E. faecalis* encounters during food preservation and processing could prime these organisms for virulence. Therefore, the expression of both stress-related and virulence genes in defined food and sub-lethal stress conditions was evaluated in three *E. faecalis* strains from clinical and food origins.

4.2.1 Hyperosmotic (NaCl) stress response

One of the oldest known food preservation methods is the reduction of water activity, or free water, in a food product through drying or the addition of water binding substances like sugar or salt. In order to survive and grow, bacteria must maintain a higher intracellular osmotic pressure compared to that of the surrounding environment, in a state called cell turgor (Sleator and Hill, 2002). This state is maintained through the carefully balanced influx and efflux of solutes, and thus water, in response to the surrounding environmental conditions (O'Byrne and Booth, 2002). Sudden changes in environmental osmolarity can disrupt this delicate balance and lead to reduced growth

and/or bacterial death. Since bacteria have a limited water activity range in with growth and or survival occurs (Sperber, 1983), lowering the water activity elongates the shelf-life of foods and can improve food safety. However, over the years, bacteria have evolved a number of different ways to deal with osmotic stress. In general, these mechanisms involve the relay of a sensed change in environmental osmolarity through a signal transduction system, which in turn stimulates the appropriate changes in gene expression within the bacterium to either synthesize or acquire compatible solutes, or release these solutes to combat changes in cell turgor and enzyme stability (Jordan *et al.*, 2008; Sleator and Hill, 2002). Often, these stress responses are coupled to changes in virulence gene expression, and thus, this aspect of the response of *Enterococcus faecalis* to osmotic stress was investigated.

The first group of genes investigated included those involved in the general stress response, which function as both chaperones and proteases and have been demonstrated to play a role in virulence in other organisms. These genes include the general stress genes, *dnaK* and *groEL*, as well as the class III heat shock genes, *ctsR* and *clpPBCEX* and the general stress gene *gls24*.

In response to a sub-lethal osmotic stress with 6.8% NaCl, all three *E. faecalis* strains (TMW 2.63, 2.629 and OG1RF) similarly up-regulated both of the class I heat shock genes *dnaK* and *groEL*. While *dnaK* transcription was up-regulated by a factor of 2.9, *groEL* transcription was up-regulated by a factor of 4.8 to 4.9 (Table 7). This correlates with a previous proteomic investigation conducted by (Flahaut *et al.*, 1997), in which a sub-lethal 6.5% NaCl stress induced *E. faecalis* DnaK and GroEL (protein) expression by factors of 2.9 and 4.8, respectively, which implies that perhaps the relative level of transcriptional up-regulation of these genes is also reflected on the

translational level and that these heat shock genes are also up-regulated in response to hyperosmotic stress.

The class III heat shock genes and their negative regulator, *ctsR*, were also generally up-regulated in response to sub-lethal NaCl stress for all three *E. faecalis* strains. As with the general stress response genes, *dnaK* and *groEL*, these genes help to stabilize and degrade misfolded proteins (enzymes) during stress, and thus, the observed up-regulation of all these genes in response to hyperosmotic stress is not unexpected. However, it is interesting to note that both *clpP* and especially *clpX* were highly up-regulated in response to hyperosmotic stress. Frees et al (Frees *et al.*, 2003; Frees *et al.*, 2005) reported that in *Staphylococcus aureus*, both *clpP* and *clpX* are more involved in virulence than in stress tolerance, and function by regulating α-haemolysin production (as well as several other extracellular virulence factors) through the *sar/agr* regulatory network. Deletions of either *agr*, *clpP* or *clpX* resulted in a similar repression in α-hemolysin production, which led the authors to propose that these two systems are similarly linked to the regulation of this virulence factor. A similar *agr* regulatory system has also been identified in *E. faecalis*, named *fsr*, and regulates the expression of both gelatinase (*gelE*) and a serine protease (*sprE*), which have also been identified as extracellular virulence factors (Qin *et al.*, 2000). Thus, *clpX* and *clpP* may also play a role in *E. faecalis* virulence through a similar mechanism.

An interesting difference was observed between these sub-lethal stress results with *clpX* and *clpP*, and the results obtained in the initial experiments with *E. faecalis* strains TMW 2.63 and OG1RF subjected to a 6.5% NaCl hyperosmotic stress. In the initial experiments, *clpP* and *clpX* were not up-regulated to the same extent as that observed with sub-lethal osmotic stress, although exponential phase cells generally expressed these two genes at a higher level than stationary phase cells. In fact, the

relative expression levels of these genes indicate that they are down-regulated in response to hyperosmotic stress, which contradicts the results obtained during the sub-lethal stress experiments. The difference in expression observed between exponential and stationary phase cells is not unexpected, as the transition to stationary phase is triggered by starvation stress, and the stationary phase bacterial cells have already initiated an increase in the expression of genes involved in the stress response. However, the disparity between the initial and sub-lethal osmotic stress results requires explanation. The sub-lethally stressed cells were grown to late exponential phase in BHI broth, which is richer in composition and preferential for *E. faecalis* growth (see growth curves in LB and BHI broths, Figures 10 and 11), while in the initial experiments, cells were grown to late exponential phase and stationary phase (24 h) in LB medium, which lacks components present in BHI broth and does not promote as much *E. faecalis* growth. Thus, the LB medium itself could play a role in eliciting a stress response in *E. faecalis*, and the lack of *clpP* and *clpX* up-regulation under these conditions is attributed to similar levels of stress between the LB control and 6.5% NaCl environments.

A similar divergence in the osmotic response was observed for the last general stress gene, *gls24*. While *gls24* was not significantly expressed in response to sub-lethal osmotic stress in BHI broth, it was significantly up-regulated in exponential phase *E. faecalis* osmotically stressed cells in LB broth. This indicates that in a nutrient-rich medium, hyperosmotic stress does not induce *gls24* expression, while the combination between nutrient stress and osmotic stress does induce *gls24* expression. In a study conducted by (Sezonov *et al.*, 2007) in which limitations on *Escherichia coli* growth in LB broth were investigated, the authors found that while this medium supported *E. coli* growth to an OD_{600} value of 7, actual steady-state growth ended at an OD_{600} value of

0.3. Steady-state growth in depleted LB medium unable to support further *E. coli* growth was restored upon the addition of glucose. Indeed, proteomic studies have found that *E. faecalis* up-regulates the expression of Gls24 during glucose starvation (Giard *et al.*, 1997; Giard *et al.*, 2000; Giard *et al.*, 2001), which supports the results in the current study. Thus, *gls24* expression observed in the initial hyperosmotic stress study in LB medium can be attributed to glucose stress rather than sub-lethal hyperosmotic stress, and indicates that hyperosmotic stress does not pre-condition *E. faecalis* for *gls24*-mediated virulence.

On the other hand, the expression of two surface protein encoding virulence genes, *efaA* and *ace*, was significantly up-regulated in response to sub-lethal hyperosmotic stress in all three *E. faecalis* strains. Both of these genes have been identified during analysis of serum from patients suffering from *E. faecalis*-associated infections, which indicates that these two surface proteins are expressed during the infection process (Lowe *et al.*, 1995; Rich *et al.*, 1999). Additionally, the influence of *efaA* on *E. faecalis* virulence has already been investigated in a study conducted by Singh et al. (Singh *et al.*, 1998), where prolonged survival was observed in a mouse peritonitis model infected with an *E. faecalis efaA* mutant compared to wild type. Studies conducted to determine physiological signals which could influence the expression of these two genes revealed that while *efaA* expression is increased upon exposure to serum (Lowe *et al.*, 1995) and acts as a high-affinity manganese permease in the absence of free manganese (Mn^{2+}) (Low *et al.*, 2003), *ace* is expressed in response to the presence of ECM molecules including collagen and laminin (Hall *et al.*, 2007; Nallapareddy and Murray, 2006). However, the influence of other environmental stresses on the expression of these virulence genes has not been evaluated, and in the current sub-lethal stress model, these two virulence genes are significantly up-regulated

in response to hyperosmotic stress. This observation can in part be explained based upon the results of a study conducted by Bourgogne et al. (Bourgogne *et al.*, 2006), which indicate that *efaA*, and perhaps *ace*, transcription is partially influenced by *fsrB* (also up-regulated under these conditions), which in turn is possibly regulated by *clpP* and *clpX* due to the similarity between the *agr* network in *S. aureus* and the *fsr* network in *E. faecalis* discussed earlier (Frees *et al.*, 2003; Frees *et al.*, 2005). However, the fact remains that *efaA* and *ace* expression is significantly up-regulated in response to hyperosmotic stress, and thus, this food preservation technique may pre-condition *E. faecalis* for *efaA*- and *ace*- mediated virulence.

One of the more intensively studied *E. faecalis* virulence genes, *gelE*, and its regulator, *fsrB*, were also both significantly up-regulated in response to sub-lethal hyperosmotic stress, which correlates with the *efaA* and *ace* results discussed in the previous paragraph. The expression of *gelE*, which encodes a metalloprotease called gelatinase, is, under normal circumstances, regulated by the product of *fsrB(D)*, called GBAP, in a quorum sensing manner (Nakayama *et al.*, 2001). Both genes are required for gelatinase production. However, these results indicate that their transcription is also significantly induced in response to hyperosmotic stress. As previously mentioned, the *agr* regulatory network in *S. aureus* bears similarities to the *fsr* regulatory network in *E. faecalis*, and its regulation is intertwined with both *clpP* and *clpX*, which are in turn, regulated in response to environmental stress. The results of the current study imply a connection between *fsr* regulation and the *E. faecalis* stress response, which raises questions regarding the roles that both *clpP* and *clpX* play in *E. faecalis* pathogenicity. However, the involvement of both *fsrB* and *gelE* in *E. faecalis* virulence has been extensively investigated in a number of animal models, and the absence of either gene led to attenuated virulence (Engelbert *et al.*, 2004; Mylonakis *et al.*, 2002; Qin *et al.*,

2000; Sifri *et al.*, 2002). As gelatinase is an excreted molecule that appears to disrupt complement mediated opsonization in human serum and thus formation of the membrane attack complex (Park *et al.*, 2007), yet is also important for translocation of *E. faecalis* across a cell monolayer (Zeng *et al.*, 2005), the hyperosmotically-induced accumulation of gelatinase in food products prior to consumption could aid in the survival in and dissemination of *E. faecalis* from the digestive tract to cause systemic infection.

Finally, the relative expression levels of two genes in the cytolysin operon, *cylB* and $cylL_L$, demonstrated both growth phase and environmental regulatory influences in response to hyperosmotic stress. *cylB* was significantly up-regulated in late log phase cells from both the sub-lethal stress experiments in BHI broth (11.2 fold) and the environmental stress experiments in LB broth (2.2 fold), while stationary cells from the latter experimental type significantly down-regulated the expression of this gene. Late exponential phase cells exposed to hyperosmotic stress in LB broth also up-regulated $cylL_L$, which is co-transcribed with *cylB*, to a lesser extent. Haas et al. (Haas *et al.*, 2002) reported that induction of the cytolysin operon begins at a cell density of 10^{-7} in BHI via a quorum-sensing mechanism, while Day et al. (Day *et al.*, 2003) found that at higher cell densities, cytolysin expression is environmentally influenced (i.e. induced by anaerobic conditions). As late exponential phase cells were above the cytolysin induction density at the time of exposure to this environment in both BHI and LB broths and relative expression levels were compared to normal growth conditions in both BHI and LB broths, the observed significant increase in *cylB* (and $cylL_L$) expression can be attributed to *E. faecalis'* response to a hyperosmotic environment. At this point, the two subunit precursors of cytolysin, $CylL_S$' and $CylL_L$', bind, are modified by CylM and transported out of the cell by CylB, where they are converted to

an active form ($CylL_S$" and $CylL_L$") by CylA. Studies have shown that a threshold concentration of $CylL_S$" is needed to maximally induce cytolysin expression over a baseline level (Coburn *et al.*, 2004), and once the available pool of $CylL_S$" is reduced, the production of cytolysin is repressed. Therefore, the significant down-regulation of cytolysin production observed in stationary phase cells in the different environments in comparison to LB probably reflects a decrease in available $CylL_S$" below the level required to maintain the baseline cytolysin expression observed in LB. This mechanism is used by *E. faecalis* to regulate cytolysin expression in the presence of target cells, where during steady state, $CylL_L$" binds to $CylL_S$" to prevent induction, yet in the presence of target cells like erythrocytes, $CylL_L$" preferentially binds to the target cell, freeing $CylL_S$" to induce cytolysin production (Coburn *et al.*, 2004). Thus, the observed significant differences in *cylB* expression reflect both growth phase and environmental influences, which could become an important factor in the degree of virulence observed in *E. faecalis* strains.

E. faecalis up-regulates the expression of many genes, including those involved in virulence, in response to sub-lethal hyperosmotic stress. While the mechanisms behind the regulation of these genes under stress conditions are currently unknown, similarities to stress-related virulence responses investigated in similar organisms indicate that these mechanisms may also exist in *E. faecalis*.

4.2.2 Acid (HCl) stress response

The use of acid, a product of fermentation, to preserve food products also has a long history of use. Weak organic acids such as those produced during fermentation easily cross the bacteria cell membrane in undissociated form and rapidly dissociate into protons and charged derivatives once they come into contact with the bacterial

cytoplasm, which generally has a higher pH than the external environment (Cotter and Hill, 2003; van de Guchte *et al.*, 2002). The accumulation of protons, which cannot cross the bacterial membrane in undissociated form, leads to cytoplasmic acidification, disruption of the proton motive force, inactivation of acid-sensitive enzymes, damaged proteins and DNA, and eventual death. Bacteria employ numerous mechanisms to combat the detrimental effects of internal acidification. These include using ATP-dependent proton pumps to expel excess protons and raise the internal pH, chaperone proteins and proteases to stabilize or degrade damaged enzymes, as well as DNA repair mechanisms (see Figure 28). While the determination of all acid stress adaptation mechanisms employed by *Enterococcus faecalis* is beyond the scope of the current study, the transcriptional regulation of genes that encode chaperones and proteases, as well as virulence genes whose regulation is sometimes closely tied to the stress response, was evaluated in three *E. faecalis* isolates.

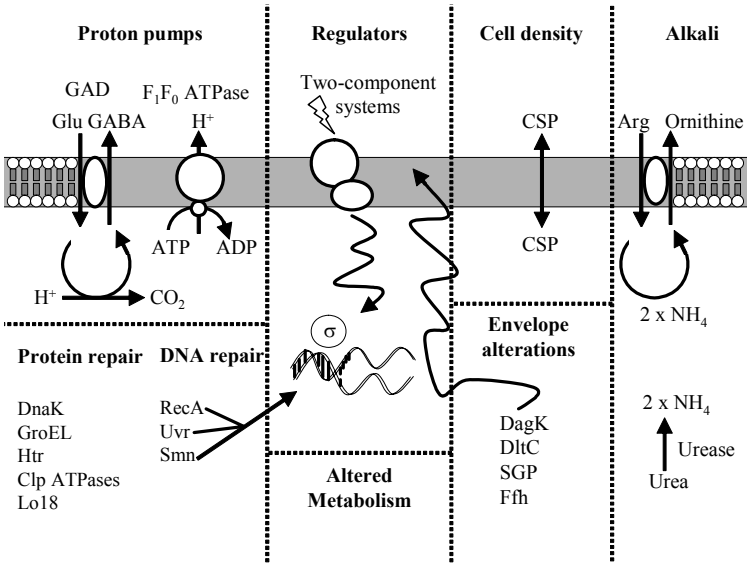

Figure 28. Graphical presentation of the mechanisms of resistance available to Gram positive bacteria in response to acid stress. These have been divided into eight categories, and a number of examples are demonstrated. (i) Proton pumps such as the F1F0ATPase or that utilized by the GAD system bring about an increase in internal pH. (ii) Proton repair involving chaperones, proteases, and heat shock proteins results in the protection of proteins or their degradation if damaged. (iii) DNA damaged as a consequence of a low internal pH can be repaired through the excision of errors or the restarting of stalled replication forks. (iv) The involvement of regulators such as 2CSs and sigma factors can induce minor or global responses. (v) Cell density affects cell-to-cell communication. (vi) Cell envelope alterations can protect cells by changing architecture, composition, stability, and activity. (vii) The production of alkali by the ADI or urease system increases the internal pH of the cell. (viii) Metabolic properties can be altered. Figure and text are from (Cotter and Hill, 2003).

As in the previous section, the first group of genes investigated included chaperones and proteases involved in the general stress response. These included the well characterized *dnaK* and *groEL* general stress chaperones, the class III heat shock response genes, *clpPBCEX*, their negative regulator, *ctsR*, and the general stress gene *gls24*.

Both *dnaK* and *groEL* were significantly up-regulated in response to sub-lethal acid stress by all three *E. faecalis* isolates examined (TMW 2.63, 2.629 and OG1RF). Induction levels for these two genes were analogous to those observed in response to the sub-lethal hyperosmotic challenge discussed in the previous section. While *dnaK* was up-regulated by a factor of 2.2–2.5, *groEL* was up-regulated by a factor of 4.4–4.9. Similar levels of induction were also observed on the proteomic level in response to acid stress (Flahaut *et al.*, 1996c), which indicates that these two genes are comparable in their transcriptional and translational rates under these conditions, and are induced at the same level in response to both sub-lethal acid and hyperosmotic stress.

On the other hand, some of the class III heat shock genes were differentially induced in response to sub-lethal acid stress compared to hyperosmotic stress. The expression levels of both *clpP* and *clpE* were analogous to those observed in response to hyperosmotic stress. However, *ctsR*, *clpC* and *clpX*, were all expressed at a lower level in response to sub-lethal acid stress, which implies that they are not as involved in this response. In general, the expression of all these genes is induced in response to various stresses, with the length and intensity of the induction contingent upon *ctsR* control (Frees *et al.*, 2007), which is, in fact, observed in the sub-lethal acid stress response under discussion. While *clpP* encodes a proteolytic unit, *clpCEX* encode various ATPases, which bind to misfolded proteins and present them to *clpP* for degradation (see introduction for a review). The exact targets of the *clp* ATPases in *E.*

faecalis are unknown at the present time, however, in other related bacteria, both *clpC* and *clpE* act to degrade the negative regulator *ctsR* in response to protein aggregation during cell stress (Kruger *et al.*, 2001), with *clpE* involved in the early stress response followed by *clpC* during the late stress response (Miethke *et al.*, 2006). Perhaps in *E. faecalis*, the response to acid stress is delayed in comparison to the hyperosmotic stress response and thus, accounts for the higher induction of *clpE* (early response). The role of *clpX* in the *E. faecalis* stress response is also unclear, and this gene was found to have different functions in various bacteria (Frees *et al.*, 2007). In the previous chapter, the significant up-regulation of both *clpP* and *clpX* in response to hyperosmotic stress presented an interesting parallel to investigations regarding these genes in *Staphylococcus aureus*, in which stress responses involving *clpP* and *clpX* are closely tied to the regulation of the virulence factor, α-hemolysin, through the *agr* regulatory network (Frees *et al.*, 2005). As the *agr* regulatory network bears similarities to the *fsr* regulatory network in *E. faecalis* (Qin *et al.*, 2000), and both the *fsrB* gene and *clpP* and *clpX* genes are all significantly up-regulated (along with gelatinase expression) in response to both acid and hyperosmotic stresses, the suggestion that a similar system of regulation exists in *E. faecalis* is a possibility. Thus, *clpP* and *clpX* may also play a role in the regulation of *E. faecalis* virulence in response to environmental stress conditions.

As in the previous chapter, *clpP*, *clpX* and *gls24* are all differentially regulated in a growth phase- and media-dependent manner in response to acid stress in LB broth, which is in contrast to results seen with the sub-lethal stress experiments in BHI broth. While exponential phase cells expressed all three of these genes at a higher level than stationary phase cells, which is expected due to the reasons presented earlier, the up-regulation of the *clpPX* genes was not as high in LB broth. This is attributed to the growth limitations imposed by LB broth due to a lack of glucose, which induces an

earlier stress response as well as *gls24* transcription (Giard *et al.*, 1997; Giard *et al.*, 2000; Giard *et al.*, 2001; Sezonov *et al.*, 2007). These results underscore the effect that environmental stress and the growth phase of the bacteria can have on gene expression. Thus, in rich food environments with properties similar to those found in BHI broth, the up-regulation of both *clpP* and *clpX* is significant and *gls24* is not a concern, while the opposite is true for more minimal food environments similar to LB broth.

It is interesting to note that the similarities in *dnaK*, *groEL*, *clp* and *ctsR* induction are reflected by the cross-tolerance acquisition between the two stresses. Exposure to a sub-lethal hyperosmotic stress provides cross-protection against a subsequent lethal stress with acid, and a sub-lethal acid stress provides cross-protection against a lethal hyperosmotic stress, which indicates that the genetic response to these stresses is similar. The involvement of stress genes included in the current study in the acquisition of this cross-protection is currently unknown, however, it appears that many other factors are involved. An analysis of protein expression profiles for the different stress conditions found that while 32 proteins are induced in response to acid stress (pH 4.8), 96 proteins are induced in response to hyperosmotic stress (6.5% NaCl) (Rince *et al.*, 2000). The overlap in protein expression between these two stress responses is also unknown at the present time, and is complicated by the fact that stress responses may not even be reflected on a proteomic level (Rince *et al.*, 2000).

The expression of the surface virulence proteins, EfaA and Ace, was also induced in response to sub-lethal acid stress in a similar manner to that observed for hyperosmotic stress. Although the level of induction was not as high, it coincided with a similar attenuation in *clpX* expression. This point is interesting as, in accordance with the earlier proposition that a link exists between *clpP/clpX* and the *fsr* regulatory network, *efaA* and *ace* (to some extent) are regulated by *fsrB* (Bourgogne *et al.*, 2006).

In *S. aureus*, the *agr* regulatory network, which is a homologue of the *fsr* system in *E. faecalis*, also controls the expression of surface proteins that allow this organism to bind to host fibrinogen, fibronectin, and collagen during infection (Frees *et al.*, 2005). Protein A is a *S. aureus* surface protein encoded by *spa* and is negatively regulated by *agr*. While the absence of *clpP* had no effect on the level of expression of Protein A, Frees et al. (Frees *et al.*, 2005) found that *clpX* is required to remove the inhibitory effect of *agr* on Protein A expression. Additionally, the level of Protein A expression reflected the concentration of ClpX, which is similar to the results observed with both *ace* and *efaA* in *E. faecalis* when the results of the hyperosmotic stress response are compared to those of the acid stress response. These systems are highly conserved in low GC Gram positive bacteria, and a homologous system of *fsr/clpX* regulation of surface protein expression may also exist in *E. faecalis*. Thus, the *E. faecalis* sub-lethal acid stress response appears to be closely tied to virulence mechanisms involving sensing and binding to host tissues.

It is interesting to note that in the initial acid stress experiments in LB broth, *efaA* expression was down-regulated by both *E. faecalis* TMW 2.63 and OG1RF equally for both exponential and stationary phase cells. Again, this result may reflect the growth limitations of the LB broth and cells in both growth phases were in physiological states similar to late stationary phase.

Lastly, the expression of the excreted proteases, gelatinase and cytolysin, is up-regulated in response to acid stress. The change in induction level for the genes encoding these proteases between the hyperosmotic and acid stress responses reflects similar changes in both *fsrB* and *clpX* expression. While *fsrB* regulation of *gelE* has been extensively characterized, including its involvement in *E. faecalis* virulence, a link between *clpX* regulation and cytolysin expression is undocumented. Further research is

required to determine if this correlation and proposed regulatory involvement of *clpX* is valid, however, the fact remains that the genes for these two well-documented virulence factors are significantly up-regulated in response to acid stress.

Acid stress significantly induces the expression of a number of stress response and virulence genes, which may prime *E. faecalis* for virulence before it even enters the host. As acidification is an application commonly used in the food industry for food preservation, this effect on *E. faecalis* gene expression should be considered during the design of food processing conditions so that sub-lethally stressed populations of cells are no longer a concern.

4.2.3 Heat stress response

The application of high temperatures to eliminate bacteria in food products (i.e. pasteurization, sterilization) is a common practice in food processing both in commercial and private arenas today. High temperature processing affects bacteria by causing protein denaturation and aggregation, destabilization of macromolecules (i.e. ribosomes, nucleic acids, enzymes), as well as membrane damage (Abee and Wouters, 1999; van de Guchte *et al.*, 2002). Bacteria have evolved many mechanisms to counteract the results of heat stress. These include the accumulation of osmolytes to protect macromolecules from the effects of heat, the formation of spores, and the expression of heat shock proteins (HSP), which either act as chaperones to stabilize, (re)fold, and disaggregate proteins or as proteases to degrade misfolded proteins. A number of these HSPs have been identified and characterized in low GC Gram positive bacteria, including the model organism *Bacillus subtilis* (see (Schumann, 2003) for a full review). In *B. subtilis*, HSPs are divided into different classes according to regulator and function. The class I HSPs, which include the DnaK and GroEL

chaperones, are controlled by the HrcA/CIRCE system while the class III HSPs, which include chaperone and protease proteins encoded by *clp*, are negatively regulated by CtsR (Laport *et al.*, 2004). So far, only the class I HSPs, DnaK and GroEL, have been characterized in *Enterococcus faecalis*. Since the bacterial stress response is closely tied to virulence in a number of other organisms, the effect of sub-lethal heat stress on the expression of stress and confirmed virulence genes in *E. faecalis* was determined.

The first group of genes examined included both the class I and class III HSPs discussed in the previous section. These include the chaperones *dnaK* and *groEL*, the protease, *clpP*, the chaperones, *clpBCEX*, their *ctsR* regulator, and the general stress gene, *gls24*. In comparison to both the hyperosmotic and acid stress responses, all of these genes were transcribed at even higher levels in response to heat stress, with the exception of *gls24*, which was expressed at very low levels under these conditions. While this increase in *dnaK* and *groEL* expression was also observed in earlier studies on both transcriptional (Laport *et al.*, 2004) and proteomic (Flahaut *et al.*, 1996c) levels, the class III HSP response has not been evaluated in *E. faecalis* until now. The significant increase in the transcription of these HSPs in response to heat stress is not unexpected, as these proteins were originally identified for their involvement in the heat shock response. Obviously, they are all required by *E. faecalis* to counteract the detrimental effects of high heat, yet not all of them were required or as highly induced in response to the other stresses discussed thus far. It is interesting to note that *clpB*, which was not up-regulated in response to either hyperosmotic or acid stress, is induced in response to heat stress. Although its function in *E. faecalis* is unknown, (Frees *et al.*, 2007) propose that since ClpB lacks a ClpP interaction domain, it primarily acts as a chaperone and works to solubilize protein aggregates. However, in *Listeria monocytogenes*, ClpB does not play a significant role in stress resistance, although it is

up-regulated in response to heat stress and is involved in the development of thermotolerance to a lethal heat stress (Chastanet *et al.*, 2004). Instead, it is involved in *L. monocytogenes* virulence, demonstrated by the nearly 100-fold difference in 50% lethal dose (LD_{50}) values between wild type and *ΔclpB* strains in a mouse model in which the virulence of the *ΔclpB* strain was highly attenuated. Both *clpP* and *clpX* were also significantly up-regulated under these conditions, which should be taken into consideration due to their possible involvement in gelatinase-mediated virulence (see previous chapters for a full explanation).

In addition to the HSPs, the expression of two virulence genes that encode the surface proteins EfaA and Ace were significantly up-regulated in response to heat stress. While the level of *efaA* transcription was homologous to that observed in response to acid stress, *ace* transcription was highly up-regulated under these conditions. Earlier studies found that Ace was only expressed at an elevated temperature of 46°C and not at the physiological temperature of 37°C (Nallapareddy *et al.*, 2000b; Nallapareddy and Murray, 2006; Xiao *et al.*, 1998), which explains the significant amount of *ace* transcription observed at the sub-lethal heat stress temperature of 51°C. Even though *in vitro* studies failed to observe Ace expression at physiological temperatures, serum from endocarditis patients contained antibodies to this surface protein, which indicates that it is expressed under physiological conditions during infection (Nallapareddy *et al.*, 2000b). Ace mediates *E. faecalis* adhesion to ECM proteins, including collagen and laminin (Hall *et al.*, 2007; Nallapareddy *et al.*, 2000a), which comprise the basal lamina layer underlying the gastrointestinal epithelium. Its high level of induction in response to sub-lethal heat stress could prime *E. faecalis* for virulence if consumed by a host with a compromised gastrointestinal epithelium.

Finally, the expression of both *gelE* and *cylB* which encode for the extracellular proteases gelatinase and cytolysin, respectively, was significantly up-regulated in response to sub-lethal heat stress. The involvement of both these proteases in *E. faecalis* virulence is well established in both *in vitro* and *in vivo* infection models. While gelatinase functions to both disrupt complement-mediated signaling in human serum (Park *et al.*, 2007) and aid in *E. faecalis* translocation (Zeng *et al.*, 2004; Zeng *et al.*, 2005), cytolysin acts to lyse both prokaryotic and eukaryotic cells (Coburn and Gilmore, 2003; Coburn *et al.*, 2004). Knockout mutants for genes involved in the regulation of both these proteases have attenuated virulence in animal models of infection. The fact that these are released into the extracellular food environment where they can accumulate in response to various environmental stresses (like heat), may contribute to the pathogenicity of *E. faecalis*.

Based upon these results, sub-lethal heat stress significantly induces the expression of a number of both stress and virulence related genes to even higher levels than those observed in response to hyperosmotic and acid stresses. Thus, this food processing application may also prime *E. faecalis* for virulence before it enters the host.

4.2.4 Nitrite stress response

Nitrite is a food additive whose use in foods as a preservative has been in use for centuries (Cammack *et al.*, 1999). This chemical has several functions in foods, which include flavor enhancement through the inhibition of off-flavor development associated with spoilage bacteria, color enhancement through its interaction with myoglobin in meats to give the characteristic pink color to these products, and finally, nitrite inhibits the growth of both spoilage and pathogenic bacteria in foods. One of the major bacteria of concern in this regard is *Clostridium botulinum*, which produces a

potent neurotoxin under anaerobic conditions and is inhibited by the presence of nitrite. The mode of action for nitrite against *Enterococcus faecalis*, and bacteria in general, is unknown and the presence of this bacteria-inhibiting compound in both meat and cheese products, which are both also commonly associated with *E. faecalis*, made nitrite stress another important point to consider in the analysis of food environmental stresses contributing to *E. faecalis* virulence gene expression.

The first group of genes examined included those encoding class I and class III HSPs, as well as the general stress and virulence gene, *gls24*. All of these genes were significantly up-regulated in response to nitrite stress, although the level of up-regulation observed for *dnaK* and *groEL* varied between *E. faecalis* TMW 2.63 and the other two *E. faecalis* strains. As the mechanism of action for nitrite is unknown, aside from the fact that it inhibits bacterial survival in foods, one can only speculate at the reasons behind these differences. On the other hand, nitrite appears to elicit a similar stress response as that observed for heat stress for all the class I and class III heat shock genes. The one difference between these two stresses is the significant up-regulation of *gls24* expression induced by nitrite stress. This protein is expressed in response to glucose starvation (Giard *et al.*, 1997), oligotrophy (Hartke *et al.*, 1998), and $CdCl_2$ stress, although the survival of a *gls24* mutant did not change in response to these or other stresses (Giard *et al.*, 2000), which indicates that perhaps another level of regulation is involved in these *E. faecalis* stress responses. Additionally, a *gls24* isogenic knockout mutant was highly attenuated in virulence when compared to its wild type in both a mouse peritonitis model (Teng *et al.*, 2005) and a rat endocarditis model (Nannini *et al.*, 2005), which indicates that this gene plays a role in *E. faecalis* virulence. Thus, nitrite stress induces *gls24* expression, which may contribute to its pathogenicity prior to host interaction.

Not only are all of the stress genes significantly up-regulated in response to nitrite stress, but transcription of the genes encoding the surface proteins, EfaA and Ace, is also significantly up-regulated in response to this stress. This increase appears to follow a similar increase in *clpX* transcriptional up-regulation that bears similarities to the *agr/clpX* mediated regulation of Protein A (also a surface protein) expression in *Staphylococcus aureus* (Frees *et al.*, 2003; Frees *et al.*, 2005). Both EfaA and Ace are expressed during *E. faecalis* infections (Lowe *et al.*, 1995; Nallapareddy *et al.*, 2000b), and appear to mediate target sensing and adhesion to host tissues to initiate infection. While EfaA is part of a Mn^{2+} permease that is expressed under low Mn^{2+} conditions similar to those found in host tissues (Low *et al.*, 2003), *ace* transcription and protein expression is induced in the presence of both collagen and laminin (Hall *et al.*, 2007; Nallapareddy and Murray, 2006), molecules that comprise the ECM underlying the gastrointestinal epithelium. The combined up-regulation of these genes in response to nitrite stress could prime *E. faecalis* for virulence once it enters the host, especially if the integrity of the gastrointestinal epithelium is compromised.

Finally, the genes encoding both CylB (cytolysin) and gelatinase are significantly up-regulated in response to nitrite stress in both *E. faecalis* TMW 2.63 and 2.629. CylB represents the membrane component of the cytolysin processing machinery, and is involved in transporting the unactivated cytolysin subunits, $CylL_L$ and $CylL_S$ out of the cell (Coburn *et al.*, 2004; Haas *et al.*, 2002). The fully activated cytolysin is active against both prokaryotic and eukaryotic cells (Coburn and Gilmore, 2003), and its accumulation in food products that contain added nitrite could not only give *E. faecalis* a selective growth advantage over other susceptible bacteria, but could also prime *E. faecalis* for virulence once it enters the host. Similarly, the production of gelatinase, which is also an extracellular protease, could enhance *E. faecalis* virulence

once it enters the host. Not only has the importance of gelatinase in the *E. faecalis* virulence process been proven in numerous animal models (Park *et al.*, 2007; Qin *et al.*, 2000; Sifri *et al.*, 2002), but gelatinase also plays a role in the ability of *E. faecalis* to translocate across an epithelial barrier (Zeng *et al.*, 2005).

Thus, sub-lethal nitrite stress induces the significant expression of both stress and virulence genes in *E. faecalis*. This could contribute to *E. faecalis* pathogenicity once it enters the host.

4.2.5 High hydrostatic pressure (HHP) stress response

The use of high hydrostatic pressure (HHP) as a food preservation method is becoming a common alternative to heat treatment as the consumer market increases its demand for minimally processed, high quality foods with an extended shelf-life. One of the major advantages of HHP is its ability to eliminate spoilage and pathogenic bacteria, as well as foodborne viruses, without changing the quality of the fresh food product through the destruction of nutrients, flavor compounds and texture seen with high temperature treatments (Buchheim, 2000; Considine *et al.*, 2008; San Martin *et al.*, 2002). This technique is already commercially applied for the treatment of oysters (Calci *et al.*, 2005), cooked pork ham, raw smoked pork loin (Fonberg-Broczek *et al.*, 2005), milk (Rodriguez *et al.*, 2005), several types of seeds (Wuytack *et al.*, 2003), guacamole, and juices (San Martin *et al.*, 2002). Studies have demonstrated that HHP application affects not only membrane fluidity and function, but cellular functions related to enzyme activity, protein synthesis, growth and mechanisms associated with transcription and translation (Considine *et al.*, 2008; Pavlovic *et al.*, 2005; Vogel *et al.*, 2005).

Unfortunately, along with this new technology come additional concerns about how the treatment affects pathogenic bacteria. Studies conducted to determine the resuscitation capabilities of different foodborne pathogens after HHP treatment have demonstrated the survival and recovery of a population of the initial culture, even after treatments at pressures used by the food industry (400 MPa or more) (Bozoglu *et al.*, 2004; Ritz *et al.*, 2006; San Martin *et al.*, 2002). This was recently also demonstrated for *Enterococcus faecalis* subjected to a 400 MPa HHP treatment similar to that used in the food industry in a meat environment (Jofre *et al.*, 2007). Additionally, characteristics such as growth phase, Gram classification, strain, media (i.e. food) composition, and storage conditions following HHP treatment all contribute to the survival or recovery of HHP treated cells. If the HHP treatment does not destroy all of the bacterial contaminants, it will have induced a stress response, which has been demonstrated to include the expression of a variety of virulence associated genes (Abee and Wouters, 1999; Arnold *et al.*, 2007; Henderson *et al.*, 2006; Rowley *et al.*, 2006). Besides eliminating problematic bacteria, insufficient HHP treatment could enhance the probability of consumers developing foodborne illnesses after eating HHP treated foods. Due to the limited knowledge available about the virulence gene regulation by pathogenic bacteria subjected to HHP treatment, it is necessary to study this aspect of bacterial gene regulation in *E. faecalis*.

The transcription of genes that encode class I and class III HSPs was significantly up-regulated in response to sub-lethal HHP. These genes encode a number of proteins with chaperone and protease functions that act to stabilize, (re)fold, or eliminate misfolded proteins, and break up aggregates in response to stress. While the pattern of up-regulation was different from those seen in response to the other sub-lethal stresses, it appears that all of these genes are involved in the HHP stress response

in *E. faecalis*, including *clpB*, which was not significantly up-regulated in response to hyperosmotic and acid stresses. Studies of this gene in *Listeria monocytogenes* and *Staphylococcus aureus* indicate that the product of this gene, ClpB, acts solely as a chaperone as it lacks a ClpP interaction domain, and is primarily involved in growth at high temperatures and acquired thermotolerance (Frees *et al.*, 2007). Its up-regulation in response to HHP stress indicates that in *E. faecalis*, it is also involved in chaperone functions under these conditions, although it could play a similar role in virulence as that observed in *L. monocytogenes* (see heat stress chapter (Chastanet *et al.*, 2004). Additionally, (Karatzas *et al.*, 2003) found that *L. monocytogenes* piezotolerance was increased in mutants unable to produce functionable CtsR, which led to an increase in ClpC and ClpP. As these genes are highly conserved in low GC Gram positive bacteria (Frees *et al.*, 2007; Karatzas *et al.*, 2003), it is possible that these proteins have a similar function in *E. faecalis*. Indeed, they are significantly up-regulated in response to sub-lethal HHP treatment, however additional study is needed to determine the role these proteins and the negative regulator, CtsR, play in the *E. faecalis* HHP stress response.

It is interesting to note that while all class I and class III heat shock genes are transcribed at a higher level in response to sub-lethal HHP stress compared to sub-lethal hyperosmotic and acid stresses, this does not provide cross-protection against a subsequent lethal stress with these two environmental conditions. On the other hand, exposure of *E. faecalis* to sub-lethal hyperosmotic and acid stresses does provide cross-protection against lethal HHP stress. Additionally, while sub-lethal HHP stress provides cross-protection against a subsequent lethal heat stress, the inverse application of these stresses does not, even though similar patterns and levels of class I and class III gene expression were observed in response to both of these stresses. This indicates that

factors other than the ones investigated in the current study are involved in the development of these cross-protections. Indeed, Hormann et al. (Hormann *et al.*, 2006) found that in a related lactic acid bacterium, *Lactococcus sanfranciscansis*, the HHP stress response involves overlapping subsets of stress-inducible proteins that are also induced in response to heat, salt, acid, cold and starvation stresses and no unique HHP stress response profile seems to exist for this bacterium, although similar cross-protection results were observed (Scheyhing *et al.*, 2004).

While the general stress gene involved in *E. faecalis* virulence, *gls24*, was not up-regulated in response to HHP stress, *ace*, which encodes a virulence-related surface protein, was significantly up-regulated in response to this stress. Ace is expressed in endocarditis patients during *E. faecalis* infection, and its expression is also up-regulated in response to the presence of collagen and laminin, which comprise the ECM layer underlying the gastrointestinal epithelial layer in the host (Hall *et al.*, 2007; Nallapareddy *et al.*, 2000b; Nallapareddy and Murray, 2006). Studies have shown that Ace mediates *E. faecalis* adhesion to these ECM proteins (Nallapareddy *et al.*, 2000a; Rich *et al.*, 1999), which contributes to its pathogenicity. Although elevated heat (46°C) was demonstrated to induce *ace* transcription and protein expression *in vitro* (Xiao *et al.*, 1998), the influence of other stress conditions on its expression has not been investigated. In a previous chapter, the theory that *ace* expression is mediated by *clpX* is presented due to the similarities between the *S. aureus agr* and *E. faecalis fsr* regulatory networks, and the influence of *clpX* in *S. aureus* surface protein expression of another adhesin, Protein A, through an *agr*-mediated pathway. The *E. faecalis fsr* regulatory network appears to include both the surface proteins, EfaA and Ace (Bourgogne *et al.*, 2006), whose expression is up-regulated in the absence of *fsrB*. Frees et al. (Frees *et al.*, 2003; Frees *et al.*, 2005) found that *agr*-mediated inhibition of

spa, encoding Protein A, is removed by ClpX, and the expression level changes of these two proteins are similar. This effect was also observed in *E. faecalis* in response to HHP stress, although *efaA* transcription was not significant and indicates that perhaps another level of regulation is involved in its expression during HHP stress. Nonetheless, *ace* transcription is significantly up-regulated in response to sub-lethal HHP stress, which can prime *E. faecalis* for adhesion and infection in hosts with a compromised gastrointestinal epithelium.

Finally, only the gene involved in cytolysin production, *cylB*, was significantly up-regulated in response to HHP stress. As cytolysin is an extracellular protease that is active against both prokaryotic and eukaryotic cells (Coburn and Gilmore, 2003), HHP stress-mediated induction of its expression could give *E. faecalis* a selective advantage both in the food environment and the host.

Sub-lethal HHP stress induces the expression of a number of stress and virulence genes in *E. faecalis*. While the mechanisms behind their regulation are unknown in *E. faecalis*, similarities to studies conducted in related organisms indicate that the class III HSPs including ClpP and ClpX and the regulator CtsR, may play significant roles in both piezotolerance and virulence of this bacteria.

4.2.6 Influence of quorum-sensing (supernatant)

Quorum-sensing is used by bacteria as a form of communication. Once these signals, in the form of homoserine lactones (Gram negative bacteria) or ribosomally generated peptide pheromones (Gram positive bacteria), accumulate to a certain threshold level in the environment surrounding the bacteria, specific gene transcription is initiated through various signaling cascades (Gobbetti *et al.*, 2007). This type of communication system is employed by bacteria to coordinate the expression of genes in

such a way that they are expressed under the correct conditions at the correct time to give the bacterial population a selective advantage, including in response to stress (Smith *et al.*, 2004). In the food matrix, bacteria like *Enterococcus faecalis* are not homogenously distributed and may exist in isolated food "pockets" where they can multiply. This can lead to the accumulation of quorum sensing molecules and thus, changes in gene expression. Thus, the quorum sensing response was investigated in *E. faecalis*.

One gene, *agg*, is highly involved in the quorum sensing mechanism of *E. faecalis*. The expression of *agg* was significantly up-regulated in both late exponential and stationary phase cells exposed to each respective supernatant. The significant up-regulation of *agg* observed upon exposure to the respective supernatants is not unexpected. In order to initiate conjugation, recipient cells produce sex pheromones, which are taken up by donor cells to induce the expression of *agg* and, thus, initiate the formation of mating aggregates (Hirt *et al.*, 2000). Since the initial screening for virulence genes in *E. faecalis* TMW 2.63 revealed the presence of genes for three different sex pheromones, it is possible that their respective concentrations in the late exponential and stationary phase supernatants was sufficient to significantly induce the expression of *agg*. It is interesting to note that the expression of *agg* was higher in stationary phase cells compared to late exponential phase cells, which supports the theory that a greater concentration of sex pheromone in the supernatant induced a comparative over expression of *agg*. The significance of *agg* as a virulence gene is supported by several studies, which implicate the involvement of *agg* in increased binding to renal tubular cells (Kreft *et al.*, 1992) and fibrin (Hirt *et al.*, 2000), increased binding and invasion of enterocytes (Sartingen *et al.*, 2000), as well as the internalization and survival within neutrophils to cause infection beyond the digestive

tract (Rakita *et al.*, 1999). The fact that non-pheromone expression of *agg* has also been observed in *E. faecalis* exposed to serum (Waters *et al.*, 2003b) indicates that different environments found in foods and the host digestive tract could induce the expression of this gene, and thus increase the chance of pathogenicity.

4.2.7 Influence of combined hyperosmotic and acid stresses

Although the investigation of how individual stresses affect *Enterococcus faecalis* stress and gene expression are useful, these stresses are often applied in combination in the actual food environment. As two of the most common conditions found in fermented meat products like sausages, which often contain *E. faecalis* as well, include both salt (NaCl) and acid, the combined effect of these two stresses on *E. faecalis* stress and virulence gene expression was investigated.

The genes involved in the heat stress response, *clpP* and *clpX*, as well as the general stress response and virulence gene, *gls24*, were the first group investigated. In general, the transcription of all these genes was up-regulated in response to the combined hyperosmotic and acid stresses, with higher expression levels observed for cells in exponential phase compared to stationary phase. This growth phase dependent difference is not unexpected, as stationary phase cells have already elicited a starvation response, which would result in a similar up-regulation of these stress genes when compared to the combined hyperosmotic/acid stress response, which accounts for the low level of induction observed in stationary phase cells. *clpP* and *clpX* encode a protease and a chaperone protein which act to stabilize, (re)fold or degrade misfolded proteins in response to an environmental stress (Frees *et al.*, 2007). Although these proteins were originally identified as members of the class III group of heat shock proteins, they are also expressed in response to other environmental stresses, including

the combined hyperosmotic/acid stress conditions of the current study. In *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Listeria monocytogenes*, both *clpP* and *clpX* are involved in virulence processes pertaining to biofilm formation, adhesin and α-hemolysin production, and the activity of listeriolysin O, respectively (Frees *et al.*, 2003; Frees *et al.*, 2005; Gaillot *et al.*, 2000; Gaillot *et al.*, 2001; Wang *et al.*, 2007). The fact that these genes and their functions appear to be highly conserved among low GC Gram positive bacteria indicates that these genes may be involved in similar virulence functions in *E. faecalis*, and their up-regulation in response to the combined hyperosmotic/acid stress found in fermented sausages may pre-condition *E. faecalis* for virulence.

In addition to the class III HSPs, transcription of the general stress and virulence gene, *gls24*, was also up-regulated in response to the combined hyperosmotic/acid stress. This gene encodes a generally cytosolic protein (Teng *et al.*, 2005) that is expressed under conditions of glucose starvation, and during $CdCl_2$ and bile salts stress (Giard *et al.*, 2001). Gls24 is also involved in *E. faecalis* virulence, and a *Δgls24* knockout mutant strain demonstrated highly attenuated virulence compared to its wild type *E. faecalis* strain in both mouse peritonitis and rat endocarditis models of infection (Nannini *et al.*, 2005; Teng *et al.*, 2005). Thus, the up-regulation of *gls24* transcription in response to the combined hyperosmotic/acid stress found in fermented sausages may also prime *E. faecalis* for virulence prior to its entry into the host.

Lastly, *E. faecalis* TMW 2.63 cells in exponential phase also significantly up-regulated *cylB* expression in response to a combined hyperosmotic/acid stress. This gene encodes the ABC membrane transport protein involved in cytolysin processing and excretion in *E. faecalis* (Haas *et al.*, 2002). The genes involved in cytolysin production, although not its regulation, exist in a single operon and are transcribed from

the same promoter. Activated cytolysin is an extracellular protease that targets both prokaryotic and eukaryotic cells (Coburn and Gilmore, 2003), and its presence is associated with increased virulence, which was demonstrated in both rabbit endophthalmitis and mouse models (Ike *et al.*, 1984; Jett *et al.*, 1992). The up-regulation of *cylB* in response to the dual hyperosmotic/acid stress indicates that these conditions may pre-activate this *E. faecalis* virulence mechanism in the food environment.

4.2.8 Sausage medium (MSM)

Due to the difficulty involved in the determination of *Enterococcus faecalis* stress and virulence gene expression in an actual food medium (with our specific *E. faecalis* strains), a liquid meat simulation medium (MSM) (Leroy *et al.*, 2005) was used to simulate conditions in a sausage environment under sterile conditions.

Although this medium is more complex than that used for the combined hyperosmotic/acid stress study, similar regulation of the stress genes, *clpP*, *clpX* and *gls24*, was observed. In general, all three genes demonstrated a higher level of expression in exponential phase cells compared to stationary phase cells for both *E. faecalis* TMW 2.63 and OG1RF. This result is not unexpected, as the transition to stationary phase is triggered by starvation stress, and the stationary phase bacterial cells have already initiated an increase in the expression of genes involved in the stress response. While the *clp* genes are primarily involved in the general stress response (Frees and Ingmer, 1999; Frees *et al.*, 2003; Gerth *et al.*, 1998), and may also play an as yet undefined role in virulence, *gls24* has also been shown to influence the degree of *E. faecalis* virulence in both mouse peritonitis (Teng *et al.*, 2005) and rat endocarditis (Nannini *et al.*, 2005) models. Additionally, vaccination of mice with immune rabbit

serum to Gls24 significantly decreased mortality after injection with a Gls24 producing *E. faecalis* strain (Teng *et al.*, 2005). The significant up-regulation of *gls24* expression observed in both strains of *E. faecalis* exponential phase cells in response to the MSM indicates that the conditions found in food could stimulate the up-regulation of *gls24* expression and contribute to *E. faecalis*'s conversion to pathogenicity.

Further indication that the environment found in sausages contributes to *E. faecalis* virulence is reflected by the significant up-regulation in transcription of *cylB* in exponential phase *E. faecalis* TMW 2.63 cells exposed to the MSM environment. Cytolysin expression is associated with an increased degree of *E. faecalis* virulence, which was observed in both rabbit endophthalmitis and mouse models of infection (Ike *et al.*, 1984; Jett *et al.*, 1992). While *cylB* encodes only the ABC membrane transport component of the cytolysin production machinery, it is transcribed from the same operon as all of the other cytolysin components (see introduction for a full review) and thus, the observed up-regulation of *cylB* transcription reflects that of the operon. Indeed, under optimal conditions on blood agar, *E. faecalis* TMW 2.63 grew and produced zones of clearing around each colony on this medium, which indicates erythrocyte lysis and confirms the ability of this strain to produce active cytolysin. The conditions in MSM also induce *E. faecalis* TMW 2.63 to up-regulate the production of this protease, which contributes to its conversion to pathogenicity.

Thus, the conditions found in a simulated sausage medium contribute to *E. faecalis* pathogenicity in a similar manner to the combined hyperosmotic/acid stress, which further indicates that the food environment can prime *E. faecalis* for virulence prior to its entry into the host.

4.3 *Enterococcus faecalis* virulence gene expression in response to physiological conditions associated with the host and gut transit

Although the environmental stresses encountered both in the food and during food processing can prime *Enterococcus faecalis* for virulence even before these organisms encounter the host, subsequent stresses or conditions encountered within the host itself can also contribute to *E. faecalis*' conversion to pathogenicity.

4.3.1 Media environments used to simulate physiological conditions

4.3.1.1 BHI broth

Due to the frequent association of *Enterococcus faecalis* with nosocomial infections of the heart (endocarditis), virulence gene expression in Brain Heart Infusion (BHI) Broth was determined relative to the more minimal LB medium. BHI broth contains infusions from both calf brain (12.5 g/L) and beef heart (5 g/L), and the physiological cues provided by these components may induce *E. faecalis* virulence gene expression.

efaA, an endocarditis-associated virulence factor, was significantly up-regulated upon exposure to BHI by cells in both growth phases. Studies conducted to determine the regulation of *efaA* found that expression is increased upon exposure to serum (Lowe *et al.*, 1995), and the reason for this increase in expression is due to the absence of free manganese (Mn^{2+}), which is an important micronutrient for *E. faecalis* (Low *et al.*, 2003). In BHI, although the total Mn^{2+} level is about 26 μM, only an estimated 5 μM is actually free (Tseng *et al.*, 2001), which would also cause an increase in *efaA* expression as observed in the present study. Low et al. (Low *et al.*, 2003) propose that the EfaCBA operon acts as a high-affinity manganese permease, and may play a role in *E. faecalis* virulence, as host tissues contain as little as 20nM Mn^{2+}. Thus, the absence or limited availability of Mn^{2+} may serve as an additional environmental signal for the

initialization of genes involved in virulence. The influence of *efaA* on *E. faecalis* virulence has already been investigated in a study conducted by Singh et al. (Singh *et al.*, 1998), where prolonged survival was observed in a mouse peritonitis model infected with an *E. faecalis efaA* mutant compared to wild type.

One of the more intensively studied virulence genes in *E. faecalis*, *gelE*, exhibited expression patterns that were isolate dependent. Both isolates demonstrated a significant increase in *gelE* expression in BHI compared to in LB for both late exponential and stationary phase cells. As BHI contains both heart and brain extracts while LB does not, these could provide environmental signals, which influence *gelE* expression. Studies conducted to determine the frequency with which certain virulence genes are associated with different types of infection have reported a higher incidence of the *gelE* genotype in *E. faecalis* strains associated with both endocarditis and other infections (Creti *et al.*, 2004; Eaton and Gasson, 2001). Other investigations have demonstrated a further correlation between *gelE* expression and the development of infection. Shephard and Gilmore (Shepard and Gilmore, 2002) reported a significant up-regulation of *gelE* in exponential phase *E. faecalis* cells exposed to serum (52 fold higher than in minimal medium), while Singh et al. (Singh *et al.*, 2005) found that an *E. faecalis* GelE- mutant demonstrated attenuated virulence in a rat endocarditis model compared to its wild type GelE+ counterpart. In a related study, Zeng et al. (Zeng *et al.*, 2005) demonstrated that the expression of *gelE* is important for translocation of *E. faecalis* across a cell monolayer. As the ability to detect environmental signals is an important part of establishing virulence, the up-regulation of *gelE* expression observed for both *E. faecalis* strains in the present study upon exposure to BHI broth may demonstrate an ability to detect host tissues and modulate gene expression in response.

It is interesting to note that although studies conducted to determine the regulation of *gelE* expression indicate that intact *fsr* genes are required for *gelE* and *sprE* expression, in the present study, significant up-regulation of *gelE* expression was detected in *E. faecalis* TMW 2.63, which lacks *fsrB*, upon exposure to BHI. Results after 24 hours of growth on gelatin agar also confirmed a lack of gelatinase production by *E. faecalis* TMW 2.63. However, Singh et al. (Singh *et al.*, 2005) found that *gelE* was expressed in an *fsrB* mutant after an extended period of incubation time, and caused a similar level of endocarditis in a rat model in comparison to the wild type strain, which was $gelE^{+}fsrB^{+}$. This indicates that *gelE* may be regulated by a different pathway or is expressed at a basal level, and is up-regulated in response to the presence of host tissues.

Finally, *cylB* expression was significantly up-regulated by *E. faecalis* TMW 2.63 exponential and stationary phase cells in BHI broth. This gene is part of the cytolysin operon, and encodes an ABC membrane transport protein involved in cytolysin production and excretion (Haas *et al.*, 2002). Cytolysin is a bacteriocin that is active against both prokaryotic and eukaryotic cells. Its role in virulence has been evaluated in several animal models, including rabbit endophthalmitis, and mouse models, where the presence of the cytolysin operon (and its expression) contributed to increased virulence (Ike *et al.*, 1984; Jett *et al.*, 1992). The significant level of *cylB* transcriptional up-regulation observed in the presence of BHI indicates that specific physiologically relevant signals present in this medium induce cytolysin expression. A similar response was also observed in both serum and urine (Shepard and Gilmore, 2002), which are also physiologically relevant environments associated with *E. faecalis* infections. In an elegant study by Coburn et al (Coburn *et al.*, 2004), the mechanism employed by *E. faecalis* to sense target erythrocytes and up-regulate cytolysin

expression in response was described. Thus, the environmental cues found in BHI broth contribute to cytolysin regulation in *E. faecalis*.

Based upon these results, the BHI broth environment induces virulence gene expression by *E. faecalis*. The frequency of association between *E. faecalis* and endocarditis (Fernandez-Guerrero *et al.*, 2002) made BHI broth, which contains extracts from both calf brain and beef heart, a decent candidate environment in which to investigate *E. faecalis* virulence gene expression in response to extra-intestinal sites within the host.

4.3.1.2 Porcine fecal extract (PFE)

While the investigation of various environmental components for their influence on *Enterococcus faecalis* virulence gene expression provides some insight into how these organisms respond in the host gastrointestinal tract, this cannot duplicate what actually occurs in the host. In an attempt to reproduce conditions in the host lower intestinal tract (colon) to some extent, a fecal extract was prepared from pigs, which share gastrointestinal similarities (including in bile production) to humans (Begley *et al.*, 2005).

Under these conditions, only *gls24* was significantly up-regulated in expression, with growth phase dependant differences. Exponential phase cells up-regulated *gls24* transcription to a much higher level than stationary phase cells. As Gls24 is expressed in response to glucose, starvation, bile salt and $CdCl_2$ stress (Giard *et al.*, 2001), its up-regulation in response to the PFE environment indicates that some or all of these stresses are found in this environment. The difference in *gls24* expression between cells in different growth phases is expected, as stationary phase cells in general have already elicited a glucose or starvation stress response that is absent in actively growing cells,

and thus, the relative difference in *gls24* expression between stationary phase cells in LB medium and PFE was not significant. Gls24 is a general stress protein, whose role in *E. faecalis* virulence was demonstrated in both rat endocarditis (Nannini *et al.*, 2005) and mouse peritonitis (Teng *et al.*, 2005) models of infection. Not only was an *E. faecalis Δgls24* mutant highly attenuated in virulence compared to its wild type *E. faecalis* strain, but anti-Gls24 immune rabbit serum reduced the infectivity of *E. faecalis* during infection. Thus, its up-regulation in the PFE environment indicates that conditions encountered by *E. faecalis* as it traverses the host gastrointestinal tract may contribute to the initiation of virulence.

4.3.1.3 Bile salts stress response

One of the major influences on bacterial survival in the gastrointestinal tract, in addition to low pH, is the presence of bile salts. Bile salts (or bile acids) comprise roughly 50% of bile, which is produced by the liver and partially stored in the gall bladder in humans (Begley *et al.*, 2005). Normal concentrations of bile salts in the small intestine range from 2 to 10 mM (Northfield, 1973). They are produced from cholesterol, with the simplest form termed chenodeoxycholid acid, and are conjugated to primarily either glycine or taurine prior to secretion (Figure 29). These molecules are amphipathic in nature, which means that they consist of both hydrophobic and a hydrophilic ends, and aggregate to form micelles or mixed micelles during the solubilization of lipids. In human bile, the ratio of glycoconjugated to tauroconjugated bile salts is 3:1, although this can be altered due to diet. While in conjugated form, bile salts are soluble under a wide range of physiological conditions, the unconjugated forms are not, although their inhibitory activity against bacteria is higher. The main function of bile is to aid in the digestion of fats, which are broken down through a

detergent-like mechanism that results in their emulsification and solubilization. Thus, during fasting periods between meals, the bile concentration in the intestine is very low and should be taken into consideration in regard to bacterial stress adaptation and cross-protection.

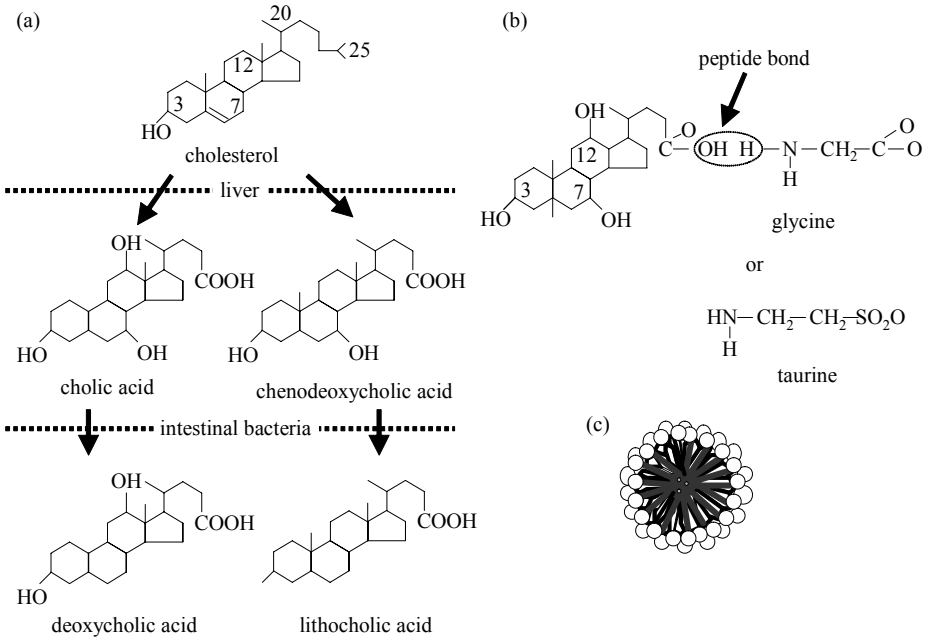

Figure 29. Bile acid chemistry. (a) Chemical structure of the major bile acids of human bile. Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized in the liver from cholesterol; these can be modified by bacterial enzymes in the intestine to form secondary bile acids (deoxycholic acid and lithocholic acid). Other bile acids are present in human bile in trace proportions but the bile acids shown compose >95% of all biliary bile acids. Redrawn from [2]. (b) All bile acids are conjugated with either glycine or taurine before secretion. The carboxyl group of the bile acid and the amino group of the aminoacid are linked by an amide bond (peptide linkage). (c) Bile acids are amphipathic and can self-associate to form polymolecular aggregates termedmicelles. These micelles can solubilize other lipids in the form of mixed micelles. Figure transcribed from (Begley *et al.*, 2005).

Due to the nature of bile salts, an obvious target for its antimicrobial activity is the bacterial membrane, although bile salts act on other sites as well. These include the structure and activity of macromolecules like RNA and DNA as well as their repair mechanisms, protein stability, and the exertion of secondary effects including acid stress (due to intracellular bile salt disassociation), osmotic stress, and oxidative stress. *Enterococcus faecalis* mutants lacking genes involved in activities related to these

targets of bile salts action (i.e. exonuclease, DNA mismatch repair, transcriptional regulator, cell wall lytic activity) were all attenuated in bile salts resistance (Breton *et al.*, 2002). Another study in which transcriptional analysis of the *E. faecalis* response to both bile salts and SDS stress was conducted, found that the majority of genes up-regulated in response to these stresses involved those with cell membrane maintenance functions (Solheim *et al.*, 2007). While a full analysis of the *E. faecalis* bile salts stress response is beyond the scope of the current study, the correlation between bacterial stress responses and virulence, the common involvement of *E. faecalis* in nosocomial infections, and its presence in foods and as a commensal enteric bacteria, made an analysis of the impact of bile salts stress on virulence gene expression necessary.

The first group of genes examined included those involved in the class I and class III heat shock responses, as well as the general stress and virulence gene, *gls24*. These genes encode for proteins with chaperone and protease functions that act to stabilize, (re)fold, or degrade proteins during an environmental stress. While the transcription of all genes (except *gls24*) was generally significantly up-regulated in response to bile salts stress, the level of this up-regulation exhibited strain differences. Differences in bile salts tolerance between bacterial strains of the same species type are not uncommon (Begley *et al.*, 2005), which could account for these isolate-related transcriptional differences in *E. faecalis*. Indeed, in contrast to results obtained for the other sub-lethal stress responses, transcriptional levels of *dnaK* and *groEL* in the current study are vastly different than those observed in proteomic studies of the bile salts stress response with a different *E. faecalis* strain, ATCC 19433 (Flahaut *et al.*, 1996b; Flahaut *et al.*, 1996c), although the same media, bile salts concentration and bile salts type (sodium cholate, sodium deoxycholate 1:1) were used. Additionally, the lack of *gls24* transcriptional induction observed in the *E. faecalis* isolates from the current

study is contrary to the results of an earlier study (albeit with a different *E. faecalis* strain, JH2-2), which found that not only was *gls24* expression up-regulated in response to an 0.08% bile salt stress (sodium cholate, sodium deoxycholate 1:1), but a *Δgls24 E. faecalis* mutant was more sensitive to bile salts challenge than the wild type strain (Giard *et al.*, 2000). Both *clpB* and *clpX* were also highly up-regulated in response to bile salts stress. The expression of these genes is tied to virulence in both *Listeria monocytogenes* and *Staphylococcus aureus* (Chastanet *et al.*, 2004; Frees *et al.*, 2003; Frees *et al.*, 2005), and, as these genes appear to be highly conserved in low GC Gram positive bacteria, ClpB and ClpX could have similar functions in *E. faecalis* virulence.

It is interesting to note that pre-adaptation to hyperosmotic, heat, nitrite and HHP stresses all provide cross-protection against a subsequent sub-lethal bile salts stress and that the inverse also applies. Begley et al. (Begley *et al.*, 2005) suggest that the acquired cross-protection is due to similar alterations in membrane function between the different stresses. Indeed, bacterial responses to all of the stresses can induce changes in the membrane to counteract the negative effects of these stresses. However, the larger concern is that if sub-lethal levels of these stresses are applied to food products, thus leaving behind a stressed and virulence gene expressing population of *E. faecalis*, these results indicate that bile salts produced in the host, although host concentrations are well above those investigated in the current study, may not be an effective defense against virulent *E. faecalis*.

Surface protein expression was differentially affected by bile salts stress compared to the other sub-lethal stress results for *efaA* and *ace* transcription. While *ace* was always expressed and *efaA* transcription was conditional, the opposite is true in response to bile salts stress, which could be attributed to the nature of bile salt antimicrobial action. Although bile salts target several different bacterial cell functions,

the initial target is the membrane (Begley *et al.*, 2005), which disrupts the regulation of membrane associated proteins. In a study conducted by Solheim et al. (Solheim *et al.*, 2007), the genome-wide transcriptional response of *E. faecalis* V583 to bovine bile compared to non-stress conditions in BHI broth was compared, and the majority of significantly up-regulated genes included those involved in membrane maintenance and energy functions. As Ace is conditionally expressed in response to the presence of ECM proteins like collagen and laminin, as well as higher temperatures (46°C; (Nallapareddy *et al.*, 2000b; Nallapareddy and Murray, 2006), which are functions not integral to membrane maintenance or energy acquisition, then the absence of its expression in the early response to bile salt stress is not unusual, although under physiological conditions, Ace expression may be induced at a later time point to these ECM proteins.

On the other hand, the transcription of *efaA* was significantly up-regulated by all *E. faecalis* strains (TMW 2.63, 2.629 and OG1RF) in response to sub-lethal bile salt stress. This gene is part of an operon of genes that encode a high-affinity manganese (Mn^{2+}) permease in *E. faecalis* expressed under low-Mn^{2+} conditions (Low *et al.*, 2003). Mn^{2+} is required in numerous cellular processes, including metabolism and oxidative stress defense (Jakubovics and Jenkinson, 2001). It also contributes to the stabilization of bacterial cell walls, which explains the significant *efaA* up-regulation observed in response to bile salts stress. However, the significance of this response is tied to the involvement of *efaA* manganese regulation in *E. faecalis* virulence. The manganese availability in host tissues can be as low as 20 nM (Krachler *et al.*, 1999), and Low et al. (Low *et al.*, 2003) propose that *E. faecalis* uses this environmental signal to infect host tissues. Indeed, a *ΔefaA* knockout mutant *E. faecalis* strain caused delayed mortality in mice compared to the wild type strain (Singh *et al.*, 1998), and

EfaA was originally identified in serum from endocarditis patients who expressed antibodies against this protein (Lowe *et al.*, 1995), which indicates that it plays a role in virulence and is expressed under physiological conditions during *E. faecalis* infection. Thus, the up-regulation of *efaA* transcription in response to bile salts stress indicates that this environmental condition (also found in the host) contributes to *E. faecalis* pathogenicity.

Of the excreted proteases, only *cylB* (cytolysin) transcription was up-regulated in response to bile salts stress. The contribution of cytolysin to *E. faecalis* virulence is well-documented. Although this virulence gene operon was initially reported to respond in a quorum-sensing manner (Haas *et al.*, 2002), subsequent studies found the environmental conditions also play a role in cytolysin regulation, including aerobiosis (Day *et al.*, 2003), serum and urine (Shepard and Gilmore, 2002), and the presence of target cells (erythrocytes; (Coburn *et al.*, 2004). The results of this study indicate that bile salt stress also induces cytolysin expression, and could contribute to the initiation of *E. faecalis* virulence in the host.

Thus, bile salt stress also induces the expression of some major *E. faecalis* virulence genes and could play a role in the conversion of *E. faecalis* from benign commensal organism to virulent pathogen in the gastrointestinal tract of susceptible hosts.

4.3.2 Intestinal epithelial cells (IEC): an *in vitro* model of *Enterococcus faecalis* infection

The increasing concern with regard to the ability of antibiotic resistant *Enterococcus faecalis* to cause nosocomial infections, and its continued use in food fermentations and as a probiotic has prompted numerous studies focused on determining its mechanism of pathogenesis. Although a number of virulence associated

genes have been identified and confirmed for virulence potential using knockout mutants for these genes and various animal models, little is known about their regulation during the actual infection process.

Several studies, including a previous study conducted in our lab, have demonstrated that *E. faecalis* is able to sense environmental changes consistent with food matrixes (Hew *et al.*, 2007) and conditions within the host (Shepard and Gilmore, 1999, 2002), and modulates virulence gene expression at the transcriptional level in response. Since foodborne *E. faecalis* appears to selectively adhere and translocate across the lower mouse intestinal epithelium to cause infection (Wells *et al.*, 1990; Wells and Erlandsen, 1991), it seems natural to assume that it senses the presence of these cells and regulates virulence gene expression in response. This phenomenon has already been observed during the characterization of cytolysin regulation in the presence of erythrocytes (Coburn *et al.*, 2004), however similar target cell "sensing" mechanisms have not been identified for other virulence-related genes.

In the present study, *E. faecalis* virulence gene regulation was investigated in response to the presence of three different cell lines – HT-29 P, HT-29 MTX and Mode-K. While all three lines are used to investigate intestinal epithelial cell function *in vitro*, the HT-29 cell lines originate from the human colon and the Mode-K cell line originates from the mouse duodenum (Vidal *et al.*, 1993). Additionally, HT-29 P cells are undifferentiated and lack the level of developmental features of columnar absorptive and mucus secreting cells present in the differentiated HT-29 MTX cell line (Lesuffleur *et al.*, 1990). Thus, several different factors were investigated for their influence on *E. faecalis* virulence gene expression: (a) species origin (human vs. mouse), (b) isolation origin (colon vs. small intestine), and (c) level of differentiation.

One of the first virulence genes analyzed for differential gene expression in response to intestinal cells was the general stress gene, *gls24*. While in general, this gene was not significantly up-regulated in response to the presence of all IECs, its expression was significantly down-regulated by both *E. faecalis* mutant strains in response to the presence of both HT-29 P and Mode-K cells, with a significant difference in its expression especially observed between HT-29 MTX and Mode-K cells. As *gls24* expression is induced by glucose starvation (Giard *et al.*, 1997) and HT-29 MTX cells consume significantly less glucose than their undifferentiated counterparts (Lesuffleur *et al.*, 1990), this may account for the observed difference in *gls24* expression in response to the different cell lines. However, in this model, *gls24* does not appear to be up-regulated in response to the presence of IECs within the first 30 min of contact.

A similar pattern of regulation was observed for the surface virulence factor, Ace. While the expression of this virulence gene was not significantly up-regulated in response to any of the IECs, its expression was generally significantly lower in the presence of Mode-K cells compared to both HT-29 cell lines. Studies have found that *E. faecalis* up-regulates Ace expression in response to the presence of various extracellular matrix proteins (ECMs), including collagen types I and IV (Hall *et al.*, 2007; Nallapareddy and Murray, 2006) and that this expression coincides with an ability to adhere to these ECMs. As all of the IECs exist as a monolayer *in vitro* and lack the collagen and laminin-rich ECM layer (basal lamina) normally present *in vivo*, this difference in *ace* expression cannot be accounted for based upon differences in collagen content between human and mouse IECs and must be influenced by other, as yet undefined, factors. However, like *gls24*, *ace* transcription is not up-regulated in the presence of IECs in this model of *E. faecalis* infection.

On the other hand, *efaAfs*, which encodes another surface protein associated with *E. faecalis* virulence, was significantly up-regulated in response to the presence of all IECs within the first 30 minutes of contact. EfaA was first identified by antigen analysis of serum from endocarditis patients (Lowe *et al.*, 1995). The gene for this protein encodes a solute binding protein receptor that is part of an operon of genes which includes *efaC*, an ATP-binding protein, *efaB*, a hydrophobic transmembrane protein, and *efaR*, which regulates the expression of *efaCBA* (Low *et al.*, 2003). Together, these genes comprise an ABC-type metal ion transport system specific for Mn^{2+}, whose transcription is induced under conditions of low Mn^{2+} availability. In host tissues, Mn^{2+} availability is estimated to be as low as 20 nM (Krachler *et al.*, 1999), which would induce the *efaAfs* transcription observed in the present study. Manganese is an important co-factor for a number of different processes in the bacterial cell, including glycolysis, amino acid metabolism and the oxidative stress defense (Jakubovics and Jenkinson, 2001). Interest in the involvement of Mn^{2+} transport systems and bacterial virulence has led to the identification of several similar systems in Salmonella Typhimurium and various oral streptococci including *Streptococcus mutans* (Kitten *et al.*, 2000; Paik *et al.*, 2003), *Streptococcus pyogenes* (Janulczyk *et al.*, 1999), and *Streptococcus gordonii* (Kolenbrander *et al.*, 1998). In these bacteria, this type of transport system appears to contribute to the virulence process by enabling these organisms to survive oxygen radical conditions inside macrophages. However, for *E. faecalis*, Low et al. (Low *et al.*, 2003) propose that *efa* Mn^{2+} regulation may be involved in this bacteria's ability to sense target cells within the host. Additionally, an *E. faecalis* knockout mutant for *efaAfs* showed delayed mortality in a mouse model, although infecting organisms were later recovered from the spleen of dead mice (Singh *et al.*, 1998), which indicates that the absence of *efaAfs* does not affect the ability of *E.*

faecalis to translocate across the intestinal epithelium to cause disease. Indeed, in the present study, all *E. faecalis* isolates significantly up-regulated *efaA* in the presence of IECs within the first 30 minutes of contact, which indicates that *E. faecalis* senses the presence of these target cells and up-regulates *efaAfs* expression in response.

Finally, the expression of excreted proteases was examined for IEC-mediated induction. The expression of the gene encoding for the cytolysin transporter, *cylB*, was not expressed in response to the presence of IEC cells, although in a previous study, the expression of cytolysin was significantly up-regulated in response to the presence of another type of target cell, the erythrocyte (Coburn *et al.*, 2004), which implies that IECs are not a target for cytolysin. Instead, the presence of IECs significantly initiated the expression of *fsrB*, *gelE* and *sprE* in the only two *E. faecalis* isolates which possess all of these genes – TMW 2.629 and OG1RF. Gelatinase, encoded by *gelE*, is implicated as the causative agent of significant virulence in a number of different animal studies (Engelbert *et al.*, 2004; Mylonakis *et al.*, 2002; Park *et al.*, 2007; Qin *et al.*, 2000; Sifri *et al.*, 2002). The expression of *gelE* is controlled via the *fsrABC(D)* genes (Qin *et al.*, 2001) in a quorum-sensing manner, and the absence of *fsrB* eliminates the expression of both *gelE* and *sprE*. While these results were also observed with the *E. faecalis fsrB* deletion mutant used in the present study (TX5266) as well as TMW2.63, which also lacks *fsrB*, the fact that *fsrB*, and thus *gelE* and *sprE* expression was significantly up-regulated in response to the presence of all IEC types is of greater significance. A study conducted by (Zeng *et al.*, 2005) also described this additional target cell sensing phenomenon in a human *E. faecalis* isolate, which only produced gelatinase in the presence of polarized human enterocyte-like T84 cells before translocation was initiated. Thus, *E. faecalis* strains TMW 2.629, OG1RF, and the human isolate described by (Zeng *et al.*, 2005), all appear to sense target cells (IECs

and T84 cells) and express gelatinase in response. While gelatinase production appears to play a significant role in *E. faecalis* virulence, the manner in which its expression is controlled (aside from quorum-sensing), remains unknown. These results imply that in a host environment (i.e. in the presence of IECs), some *E. faecalis* strains employ an alternative method of control for gelatinase expression.

As *E. faecalis* continues to be used in foods, yet remains a significant instigator of nosocomial infections, a greater understanding of the environmental conditions within the host that contribute to *E. faecalis* virulence is needed before informed decisions regarding strain usage in foods can be made. In this part of the study, the intestinal epithelium itself was investigated for its influence on *E. faecalis* virulence gene expression. While most of the virulence genes investigated did not appear to be significantly up-regulated in response to the presence of the various IECs, the transcription of both *efaAfs* and the gelatinase operon (*fsrB/gelE/sprE*) were significantly influenced by the presence of these cells. Thus, *E. faecalis* appears to sense the presence of target cells, which include intestinal epithelial cells from the human colon as well as from the mouse small intestine, and up-regulates the expression of known virulence genes in response.

4.3.3 Mouse model: an *in vivo* model of *Enterococcus faecalis* infection

Although the use of *in vitro* cell culture models offer a fast and relatively inexpensive way to investigate *Enterococcus faecalis* virulence gene regulation in a simulated host environment, *in vivo* animal models such as the mouse provide a more accurate picture of the infection process. Interleukin-10 knockout (IL-$10^{-/-}$) mice are a well-characterized mouse model used to study inflammatory bowel disease (IBD) (Balish and Warner, 2002; Kim *et al.*, 2005). These mice lack the ability to produce IL-

10, which acts to suppress or modify the inflammatory response, and develop intestinal inflammation upon exposure to normal commensal bacteria, with a few exceptions, in contrast to its wild type (WT) counterpart (Kim *et al.*, 2005; Rennick *et al.*, 1995). This inflammatory response is similar to that seen in patients with IBD, and thus, IL-10$^{-/-}$ mice provide a surrogate model to study the etiology of this type of disease.

Several studies using IL-10$^{-/-}$ and WT mice have investigated the mechanisms behind the development of IBD using *E. faecalis* as a stimulatory agent. In IL-10$^{-/-}$ mice monoassociated with *E. faecalis*, the physical manifestation of IBD occurs between 12 and 16 weeks after monoassociation, with inflammation primarily localized to the colon (lower intestine) (Balish and Warner, 2002; Kim *et al.*, 2005; Shkoda *et al.*, 2007), which was also observed in the mice used in the current study. This response was different than that observed with *Escherichia coli*, which elicited a lower degree of inflammation that was localized to the cecum in IL-10$^{-/-}$ mice. While the mechanisms behind this specific localized inflammation are not well understood, these results demonstrate that these mice respond differently to various microbial antagonists and that the sites of infection are particular to the bacteria. Indeed, Ortega-Cava et al (Ortega-Cava *et al.*, 2003; Ortega-Cava *et al.*, 2006) demonstrated that Toll-like receptors (TLR), which recognize specific ligands or pathogen associated molecular patterns (PAMPs) (Furrie *et al.*, 2005) and induce a specific inflammatory pattern in response (Sartor, 2008), are strategically localized to different areas in the mouse gastrointestinal tract. TLR 2 recognize Gram positive and mycobacterial PAMPs and in the mouse, are highly expressed in the colon compared to the rest of the gut, and are up-regulated even further in the same locality during colitis. This supports the localized inflammation observed in the colon of IL-10$^{-/-}$ mice monoassociated with *E. faecalis*, and is of particular interest as in all of these studies, *E. faecalis* was monoassociated

with the mice via the stomach and did not elicit a significant inflammatory response in any of the tissues before the colon (Figure 30). Additionally, investigation of *E. faecalis*

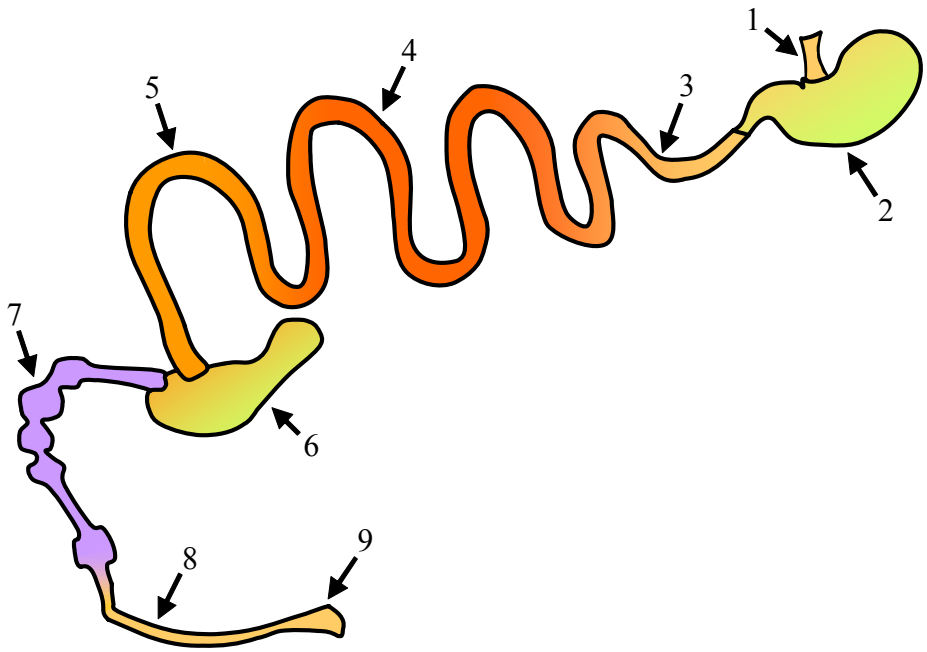

Figure 30. Gross anatomy of the mouse gasterointestinal tract from the right side. IL-10$^{-/-}$ and WT mice were monoassociated with *E. faecalis* by gastric lavage to the stomach (2). From this point, *E. faecalis* traveled through the duodenum (3), jejunum (4), ileum (5), and cecum (6) to elicit the highest inflammatory response in the ascending (7) and descending (8) colon. This diagram is modified from the National Institute of Allergy and Infectious Diseases National Institutes of Health website on mouse gross anatomy.

mediated infection in IL-10$^{-/-}$ mice indicated that the development of colitis was associated with an inability to inhibit TLR 2-mediated pro-inflammatory gene expression (Ruiz *et al.*, 2005). These responses are normally down-regulated 7 to 10 days after bacterial colonization in the normal host through the activation of inhibitory molecules like IL-10, a response which is disabled in IL-10$^{-/-}$ mice (Sartor, 2008).

Although this information provides a better understanding of the factors involved in the development of *E. faecalis* mediated disease in the host, the response of *E. faecalis* to the host environment is unknown. An initial investigation from the current study, in which the development of *E. faecalis*-mediated infection in IL-10$^{-/-}$ mice was monitored over time, demonstrated that *E. faecalis* up-regulated transcription

of the excreted metalloprotease, gelatinase, at the same time that severe inflammation occurred in the mouse (14 weeks post-monoassociation). This was not observed in WT mice at any time, and correlated with a lack of inflammation, which implies that *E. faecalis* gelatinase is involved in the development of colitis in this model. This result focused additional attention on the role that gelatinase and the genes regulating it play in the development of colitis in IL-$10^{-/-}$ mice, and further investigations in this mouse model were conducted using isogenic deletion mutants of *E. faecalis* OG1RF, although the regulation of other virulence associated genes was also determined.

While the general stress gene, *gls24*, was not significantly induced after 15 weeks of *E. faecalis* monoassociation in IL-$10^{-/-}$ mice, the gene encoding the surface protein, EfaA, was significantly induced by *E. faecalis* OG1RF at this point in the infection process. This up-regulation coincided with the development of severe inflammation in IL-$10^{-/-}$ mice monoassociated with *E. faecalis* OG1RF that was not observed in WT mice, and was significantly higher than the inflammation observed in IL-$10^{-/-}$ mice monoassociated with either of the *E. faecalis* mutants. EfaA was first identified by antigen analysis of serum taken from patients with infectious endocarditis, and demonstrated some homology to an adhesin group from oral streptococci (Lowe *et al.*, 1995). Further analysis indicated that *efaA* is part of a manganese-regulated operon encoding a permease that is induced under Mn^{2+} limiting conditions, including those found in serum or human tissues where Mn^{2+} availability is around 20 nM (Krachler *et al.*, 1999; Low *et al.*, 2003) – a situation which may be exploited by *E. faecalis* during the infection of host tissues. The absence of *efaA* also caused prolonged survival of infected mice (Singh *et al.*, 1998), which indicates that it plays a role in the *E. faecalis* virulence process. In the current study with IL-$10^{-/-}$ mice, *efaA* expression was induced by all *E. faecalis* strains in the IL-$10^{-/-}$ mice compared to the WT mice, although only

E. faecalis OG1RF significantly up-regulated *efaA* expression. This graded *efaA* up-regulation correlated with differences in the degree of inflammation seen in the mice, although mice monoassociated with the *E. faecalis* mutants had significantly lower inflammation than those monoassociated with *E. faecalis* OG1RF. The least amount of *efaA* induction (which coincided with a low inflammatory response in the mouse) was seen in the *fsrB* deletion mutant, TX5266. In BHI broth, *efaA* expression was also lower in *E. faecalis* TX5264 cells compared to OG1RF in early (4 h) and entry (5 h) stationary phase, which indicates that *fsrB* plays a role in *efaA* regulation (Bourgogne *et al.*, 2006). However, whether or not this can be attributed to the different degrees of inflammation seen in the IL-$10^{-/-}$ mouse model is unclear.

In addition to the up-regulation seen with *efaA*, *ace* transcription was also induced in IL-$10^{-/-}$ mice compared to WT mice after 15 weeks of monoassociation with both *E. faecalis* OG1RF and TX5264. Ace is also a surface molecule against which antibodies were produced during infectious endocarditis (Nallapareddy *et al.*, 2000b), and is expressed in response to the presence of extracellular matrix (ECM) components including collagen types I and IV (Nallapareddy and Murray, 2006). Adhesion to various other ECM components, including laminin, fibrinogen, and collagen types II and III was also demonstrated, and could be inhibited by pre-incubation of *E. faecalis* grown at 46°C (a condition which induces Ace expression) with anti-Ace antibodies (Hall *et al.*, 2007; Nallapareddy *et al.*, 2000a). While *ace* was significantly up-regulated by *E. faecalis* TX5264 and to a lesser extent by *E. faecalis* OG1RF, this difference in expression was not reflected in the level of inflammation seen in the IL-$10^{-/-}$ mice. In fact, the opposite effect was seen, which indicates that a higher level of *ace* expression does not correlate with higher inflammation. However, the up-regulation of *ace* transcription in general could be attributed to exposure of the basement membrane, or

lamina propria, underlying the epithelial cell layer to the lumen environment, including *E. faecalis*. Balish and Warner (Balish and Warner, 2002) found that colonic lesions and cecal ulcers develop in IL-$10^{-/-}$ mice monoassociated with *E. faecalis*. This means that the normal barrier function provided by the intact epithelium is no longer available in these places and the underlying basement membrane, which is primarily composed of collagen type IV and laminin, is exposed. Thus, the ability of *E. faecalis* to sense the presence of these proteins and up-regulate *ace* expression in response to promote binding, is yet another mechanism employed by this bacteria to "sense" and bind to targets in the host. It is interesting to note that *ace* was not expressed by *E. faecalis* TX5266, which lacks *fsrB*. A study conducted by (Bourgogne *et al.*, 2006) found that transcriptional control of *ace* may be linked to *fsrB*, which supports the result observed in the current study.

Finally, the transcription of *gelE* was up-regulated in IL-$10^{-/-}$ mice to coincide with the development of severe colitis, which was not observed in IL-$10^{-/-}$ mice monoassociated with the mutant *E. faecalis* strains. This physiological effect was observed in other animal models as well, with the same *E. faecalis* strains (Engelbert *et al.*, 2004; Mylonakis *et al.*, 2002; Qin *et al.*, 2000; Sifri *et al.*, 2002; Singh *et al.*, 2005). In general, these studies found that the *E. faecalis fsrB* mutant (TX5266) was more attenuated in killing or virulence than either the *gelE* (TX5264) or *sprE* mutants, and all were more attenuated in killing or virulence than the wild type OG1RF strain. While this clearly indicates that *gelE* plays a role in *E. faecalis* virulence, the observation that the *fsrB* knockout mutant, TX5266, is even more attenuated than the *gelE* or *sprE* mutants indicates that *fsrB* plays an even larger role in *E. faecalis* virulence than *gelE*. If one considers that *fsrB* regulates the expression of numerous other genes, including the virulence genes *efaA* and possibly *ace* (Bourgogne *et al.*, 2006), this implication is

not so surprising. In the current study, while *fsrB* expression was not significant at sampling time, mRNA transcripts from both *gelE* and *sprE* were up-regulated by *E. faecalis* OG1RF and TX5264 to coincide with the development of colitis in the IL-10$^{-/-}$ mice, indicating that perhaps the majority of *fsrB* was already translated into protein (and therefore capable of initiating *gelE/sprE* transcription), and was therefore undetectable using mRNA quantification techniques.

Although studies have attempted to identify the impact of virulence genes in animal models using knockout mutants, none have analyzed the general response of *E. faecalis* to the host intestinal environment. This is especially important, as *E. faecalis* is commonly found associated with food either purposefully added for fermentation or through contamination, and one of the proposed models for the development of this type of infection is by translocation across the intestinal epithelium in susceptible hosts. IL-10$^{-/-}$ mice provide a good animal model for the study of this type of infection. Studies conducted to determine the host mechanisms which contribute to the development of IBD indicate that a genetic disruption in the maintenance of intestinal barrier function/response is involved in disease development, and the absence of the IL-10 gene elicits a similar response to bacterial infection in these mice. Therefore, one can correlate the development or etiology of intestinal disease with changes in bacterial gene expression. The results of the current study indicate that *E. faecalis* OG1RF up-regulates *efaA*, *ace* and *gelE* in the host environment, and that this correlates with the development of severe colitis in IL-10$^{-/-}$ mice. Additionally, the attenuated degree of inflammation associated with the absence of both *gelE* and *fsrB* and the related effects of these deletions on *efaA*, *ace* and *gelE* expression indicate that *fsrB* plays an even greater role in *E. faecalis* virulence than its control of *gelE* expression alone. These

observations provide a better understanding of how *E. faecalis* responds to a susceptible host intestinal environment to cause inflammation and infection.

5 Conclusion

The purpose of this study was to determine what environmental factors contribute to the pathogenicity of *Enterococcus faecalis*. While numerous studies, including the first part of the present study, have primarily focused on the identification of potential virulence factors or genes, as well as virulence gene patterns associated with *E. faecalis* isolated from different origins, current information regarding the contribution of environment to virulence gene expression is limited.

The initial analyses of virulence gene patterns indicate, simply on a genetic basis, that pathogenicity cannot be predicted based upon the number or type of virulence genes. Isolates from food, clinical (disease), and commensal origins did not possess distinctly different patterns of virulence genes, which has the logical implication that all of these isolates are originally from fecal origin. A phenotypic analysis of these virulence genes also revealed that although these genes are present, they are not always expressed. Thus, rather than basing our understanding of *E. faecalis* pathogenicity simply on their origin of isolation or the presence or absence of virulence genes, we should strive to decipher the environmental signals that control their regulation.

In the present study, all of the environments investigated which represented those encountered in foods, during food processing, and in the host, induced differential amounts of stress and virulence gene expression. Tables 5-7 summarize the virulence gene expression patterns of the strains tested under various stress conditions. Significant changes are highlighted. While the stress genes *dnaK*, *groEL*, *ctsR*, *clpP* and *clpX* are highly induced by heat, nitrite, HHP and bile salts stresses in particular, the virulence genes involved in surface protein synthesis, *efaAfs* and *ace*, have different induction patterns. On the other hand, the production of both the excreted proteases

Table 5. *Enterococcus faecalis* TMW 2.63 stress and virulence gene expression in response to food and host environmental conditions examined in initial experiments. Values in bold are significant and trends of high up-regulation are highlighted in grey.

Virulence gene expression levels of *Enterococcus faecalis* TMW 2.63 relative to LB										
	Environmental condition	Growth phase	Virulence genes							
			clpP	*clpX*	*gls24*	*efaAfs*	*agg*	*gelE*	*cylL$_L$*	*cylB*
Food	Respective supernatant	exponential			**3.7**	**–4.3** ±1.3	**2.3**	**–5.2** ±2.8	**–2.0**	**–10.0** ±4.8
		stationary			**3.4**	**–3.1** ±0.1	**10.2**	–1.9 ±0.4	**–5.8**	**–4.3** ±1.8
	Sausage medium (MSM)	exponential	**–10.1** ±4.6	**13.4** ±6.6	**2.3** ±0.6	**–2.3** ±1.1	**–4.6** ±3.0	**–1.2** ±0.1	–1.6 ±0.3	**6.6** ±2.6
		stationary	**–20.5** ±18.1	–1.3 ±0	1.9 ±0.2	**2.1** ±0.2	**–2.2** ±0.2	–1.5 ±0.2	**–3.1** ±0.4	**–15.9** ±10.8
	6.5% NaCl	exponential	**4.6** ±1.7	**–2.5** ±1.3	**11.9** ±5.6	1.5 ±0	**–4.6**	**–2.0** ±0.5	1.6 ±0.1	**2.2** ±0
		stationary	**–21.7** ±18.8	**–3.1** ±0.3	**2.3** ±0.5	**–2.9** ±0.5	–1.4	–1.7 ±0.4	**–3.0** ±0.4	**–29.1** ±3.1
	pH5	exponential	**4.7** ±2.3	**2.4** ±0.5	**3.3** ±1.3	**–3.2** ±1.0	–1.9	**2.0** ±0.4	1.2 ±0.1	**11.1** ±1.7
		stationary	**–51.9** ±25.5	**–2.2** ±0.5	1.3 ±0.1	**–3.0** ±0.4	**–2.7**	–1.9 ±0.6	**–2.4** ±0.4	**–11.6** ±3.1
	6.5% NaCl + pH5	exponential	**3.2** ±0.4	1.4 ±0.3	**9.4** ±4.5	**–2.2** ±0.7	**–3.6**	**–2.4** ±0.6	1.2 ±0.2	**2.2** ±0
		stationary	**3.1** ±1.5	**–3.0** ±1.0	**4.9** ±2.4	1.8 ±0.4	**–9.6**	**–2.2** ±0.9	**–4.1** ±1.5	**–10.5** ±5.6
Host	BHI	exponential			**–9.5**	**5.3** ±1.4	1.6	**2.3** ±0.4	**–3.5**	**8.3** ±2.9
		stationary			**2.1**	**4.7** ±3.0	**–2.1**	**3.3** ±1.8	–1.1	**3.2** ±1.0
	Porcine fecal extract (PFE)	exponential			**18.4**	**–93.7** ±87.3		**–7.0** ±5.7	**–3.1**	–1.6
		stationary			**2.1**	**–9.0** ±3.0	**–3.3**	**–2.2** ±0.1	**–3.4**	**2.9**

Table 6. *Enterococcus faecalis* OG1RF stress and virulence gene expression in response to food and host environmental conditions examined in initial experiments. Values in bold are significant and trends of high up-regulation are highlighted in grey.

Virulence gene expression levels of *Enterococcus faecalis* OG1RF relative to LB							
	Environmental condition	Growth phase	Virulence genes				
			clpP	*clpX*	*gls24*	*efaAfs*	*gelE*
Food	Respective supernatant	exponential			**–22.1**	**–2.1** ±0.4	**–3.3** ±1.3
		stationary			1.1	−1.5 ±0.5	**–3.2** ±0.7
	Sausage medium (MSM)	exponential	**38.7** ±33.7	**11.6** ±8.0	**2.4** ±0.5	−1.5 ±0.1	**–5.4** ±2.8
		stationary	−1.4 ±0.3	**2.0** ±0.6	**4.3** ±0.9	**3.0** ±0.4	**–2.7** ±0.9
	6.5% NaCl	exponential	−1.6 ±0.6	**2.8** ±1.8	**8.7** ±1.1	**–2.0** ±0.6	**–24.7** ±4.4
		stationary	**–9.2** ±0	**–2.8** ±0.7	1.9 ±0.2	**–2.9** ±0.7	**–6.5** ±1.0
	pH5	exponential	**–2.0** ±0.7	1.1 ±0.1	**3.1** ±1.5	**–2.7** ±0.9	**–25.0** ±4.2
		stationary	**57.0** ±41.8	**–2.0** ±0.1	**10.9** ±1.8	**–2.2** ±0.6	**–3.6** ±0
	6.5% NaCl + pH5	exponential	**3.1** ±0.9	**3.3** ±1.0	**11.0** ±6.8	1.4 ±0.1	**–7.1** ±4.0
		stationary	**2.4** ±1.1	1.5 ±0.7	**3.5** ±0.7	**2.9** ±1.2	**–2.3** ±0.4
Host	BHI	exponential			**–31.1**	**3.0** ± 0.7	**2.5** ± 0.5
		stationary			**–2.8**	**3.2** ± 1.2	**2.7** ± 0.5
	Porcine fecal extract (PFE)	exponential			**14.1**	**–39.0** ± 8.1	**–16.6** ± 2.7
		stationary			1.3	**–18.8** ± 11.0	**–15.2** ± 11.3

Table 7. *Enterococcus faecalis* stress and virulence gene expression in response to sub-lethal stresses encountered in the food, food processing environments and host. Values in bold are statistically significant ($p<0.05$) and trends of high up-regulation are highlighted in grey.

	Sub-lethal stress	*Enterococcus faecalis* strain	Genes													
			dnaK	*groEL*	*ctsR*	*clpP*	*clpB*	*clpC*	*clpE*	*clpX*	*gls24*	*efaAfs*	*ace*	*fsrB*	*gelE*	*sprE*	*cylB*
Food	Heat (51°C)	TMW 2.63	**10.1** ± 2.5	**23.6** ± 4.0	**8.5** ± 0.3	**14.5** ± 2.1	**3.5** ± 0.2	**6.8** ± 1.4	**11.0** ± 0.8	**19.3** ± 1.8	1.3 ± 0.2	**2.1** ± 0.5	**12.5** ± 3.1	0	0	1.5 ± 0.4	**14.8** ± 1.4
		TMW 2.629	**10.1** ± 1.8	**23.1** ± 3.9	**9.0** ±1.8	**17.0** ± 2.1	**4.5** ± 0.6	**6.1** ± 1.4	**13.0** ± 2.7	**19.1** ± 2.8	1.6 ± 0.3	**2.0** ± 0.3	**10.4** ± 2.6	**3.1** ± 0.3	**2.1** ± 0.4	1.4 ± 0.2	0
		OG1RF	**10.1** ± 1.1	**22.7** ± 3.3	**7.4** ± 0.4	**16.8** ± 1.9	**2.7** ± 0.2	**5.6** ± 1.4	**12.2** ± 1.0	**17.4** ± 1.8	2.8 ± 1.5	**3.0** ± 0.3	**13.7** ± 3.8	**3.4** ± 0.6	1.6 ± 0.4	1.2 ± 0.3	0
	Nitrite (200 ppm)	TMW 2.63	**5.9** ± 1.1	**4.9** ± 1.4	**6.6** ± 2.7	**9.5** ± 2.9	2.7 ± 0.9	**5.6** ± 1.9	**4.3** ± 1.6	**15.1** ± 2.3	2.4 ± 0.8	**3.6** ± 0.9	**9.3** ± 4.3	0	0	1.3 ± 0.2	**7.4** ± 0.6
		TMW 2.629	**12.5** ± 1.6	**11.1** ± 0.4	**6.2** ±1.5	**7.3** ± 1.3	**3.2** ± 0.8	**4.5** ± 0.3	**4.2** ± 0.4	**11.2** ± 2.3	**3.2** ± 0.7	**2.5** ± 0.6	**8.1** ± 0.7	**4.8** ± 0.4	**4.0** ± 0.2	**2.9** ± 0.8	0
		OG1RF	**11.1** ± 2.6	**9.1** ± 2.8	**5.7** ± 1.2	**9.3** ± 1.9	**3.1** ± 0.2	**4.9** ± 1.3	**4.3** ± 0.8	**12.8** ± 3.9	**3.6** ± 1.1	**2.9** ± 0.9	**7.4** ± 2.9	**4.6** ± 0.6	1.9 ± 0.5	3.4 ± 1.6	0
	HHP (80 MPa)	TMW 2.63	**8.0** ± 2.3	**12.1** ± 2.6	**9.7** ± 1.2	**8.9** ± 1.6	1.7 ± 0.6	**7.7** ± 1.4	**4.4** ± 1.1	**18.8** ± 4.1	2.1 ± 1.0	2.1 ± 0.6	**12.5** ± 0.8	0	0	1.6 ± 0.7	**14.7** ± 1.7
		TMW 2.629	**11.9** ± 2.6	**14.8** ± 2.7	**8.4** ±1.6	**10.6** ± 2.8	**3.8** ± 0.4	**7.9** ± 1.9	**7.2** ± 1.2	**15.7** ± 3.9	1.6 ± 0.5	1.6 ± 0.3	**8.7** ± 1.7	**2.7** ± 0.9	1.7 ± 0.2	1.4 ± 0.3	0
		OG1RF	**9.9** ± 1.8	**13.5** ± 3.0	**9.2** ± 0.8	**9.4** ± 1.1	**2.3** ± 0.7	**7.2** ± 1.4	**6.4** ± 1.0	**19.2** ± 3.8	2.0 ± 0.9	1.9 ± 0.6	**6.5** ± 2.1	**3.5** ± 0.9	1.6 ± 0.4	1.1 ± 0.1	0
	Salt (6.8%)	TMW 2.63	**2.9** ± 0.5	**4.9** ± 0.3	**6.6** ± 1.0	**3.5** ± 0.7	1.6 ± 0.6	**3.6** ± 1.1	**2.3** ± 0.3	**18.4** ± 2.0	1.1 ± 0.1	**5.7** ± 0.4	**6.4** ± 0.8	0	0	1.3 ± 0.1	**11.6** ± 1.8
		TMW 2.629	**2.9** ± 0.4	**4.8** ± 0.5	**5.1** ±0.9	**5.9** ± 0.5	2.2 ± 1.1	**3.2** ± 1.2	**3.3** ± 0.5	**14.0** ± 3.7	1.4 ± 0.1	**3.8** ± 0.6	**4.3** ± 0.3	**7.6** ± 3.5	**3.7** ± 0.9	1.7 ± 0.3	0
		OG1RF	2.9 ± 1.3	**4.8** ± 1.8	**5.2** ± 1.6	**4.9** ± 1.1	1.8 ± 0.6	**3.7** ± 1.4	3.0 ± 1.2	**15.6** ± 6.4	1.3 ± 0.3	**4.2** ± 0.5	**4.5** ± 1.8	**8.1** ± 2.4	**4.5** ± 0.6	1.5 ± 0.2	0
	Acid (pH 4.1)	TMW 2.63	**2.2** ± 0.1	**4.4** ± 0.3	**2.4** ± 0.3	**4.0** ± 0.2	1.2 ± 0.1	1.3 ± 0.1	**2.3** ± 0.2	**7.7** ± 1.2	1.3 ± 0.1	**2.1** ± 0	1.8 ± 0.1	0	0	1.0 ± 0	**4.5** ± 0.2
		TMW 2.629	**2.2** ± 0.1	**4.5** ± 0.1	**2.6** ±0.1	**5.0** ± 0.3	1.3 ± 0.1	**2.2** ± 0.2	**3.6** ± 0.1	**7.7** ± 0.4	1.4 ± 0	**2.9** ± 0.1	**3.7** ± 0.1	**3.4** ± 0.1	**2.3** ± 0.1	1.4 ± 0.1	0
		OG1RF	2.5 ± 0.9	**4.9** ± 2.0	**2.8** ± 0.8	**3.7** ± 1.0	1.3 ± 0.2	1.6 ± 0.5	**2.5** ± 0.5	**6.9** ± 1.8	1.4 ± 0.5	**2.8** ± 0.8	**2.1** ± 0.2	**4.3** ± 0.7	**2.4** ± 0.4	1.3 ± 0.2	0
Host	Bile salts (0.08%)	TMW 2.63	**20.6** ± 3.3	**8.2** ± 2.1	**4.5** ± 1.0	**5.6** ± 1.4	**6.4** ± 0.5	**5.5** ± 0.8	**5.2** ± 0.8	**10.5** ± 2.1	**2.0** ± 0.2	**5.5** ± 0.2	1.6 ± 0.2	0	0	1.7 ± 0.6	**4.0** ± 0.5
		TMW 2.629	**12.9** ± 3.3	6.2 ± 2.3	3.5 ±1.5	4.6 ± 1.4	**3.2** ± 0.6	**3.8** ± 0.8	**4.7** ± 0.9	**10.6** ± 2.7	1.8 ± 0.7	**5.7** ± 1.8	1.36 ± 0.41	3.79 ± 1.56	1.6 ± 0.5	1.4 ± 0.4	0
		OG1RF	**15.4** ± 4.1	**7.4** ± 2.0	**3.9** ± 1.0	**4.7** ± 1.2	**3.2** ± 0.7	**3.5** ± 0.5	**4.4** ± 1.1	**15.9** ± 2.2	1.6 ± 0.1	**4.8** ± 1.2	1.4 ± 0.1	3.5 ± 1.3	1.7 ± 0.4	1.1 ± 0	0

gelatinase and cytolysin is transcriptionally up-regulated in response to all investigated sub-lethal stresses. Gelatinase and *efaA* transcriptional up-regulation also correlated with both the presence of intestinal epithelial cells and the development of severe colonic inflammation in a mouse model of IBD, which indicates that these results translate to an actual host environment and that the state of the host (an inability to produce IL-10) influences virulence gene expression. Parallels between the *E. faecalis fsr/gelE* regulatory system and the *Staphylococcus aureus agr* regulatory system, which is in turn regulated by the level III heat shock genes *clpP* and *clpX* (Frees *et al.*, 2005), additionally suggest that stress and virulence gene expression are also closely tied in *E. faecalis*.

While these observations in combination with results from the cross-protection studies, which demonstrate that the food environment and the order of environmental challenges applied during food processing and after consumption by the host may either increase the hardiness of *E. faecalis* or contribute to its demise, support suggestions that the use of *E. faecalis* in foods and as a probiotic be restricted, one should maintain a perspective on these results. Rather than the implementation of numerous regulations regarding the use of this LAB, which also have a positive history of use in foods, several guidelines (based upon the results of this study) can be incorporated into criteria for the selection of starter cultures and probiotics as a precaution. This includes the use of *E. faecalis* strains that lack both *fsrB* (this gene appears to regulate the expression of several virulence genes including *gelE* and *efaA*) and *agg* (this gene is involved in the spread of both antibiotic resistance and virulence genes), and the regulation of the intensity and order of applied stresses (i.e. food processing conditions) to avoid pre-conditioning *E. faecalis* for virulence.

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7 Appendix

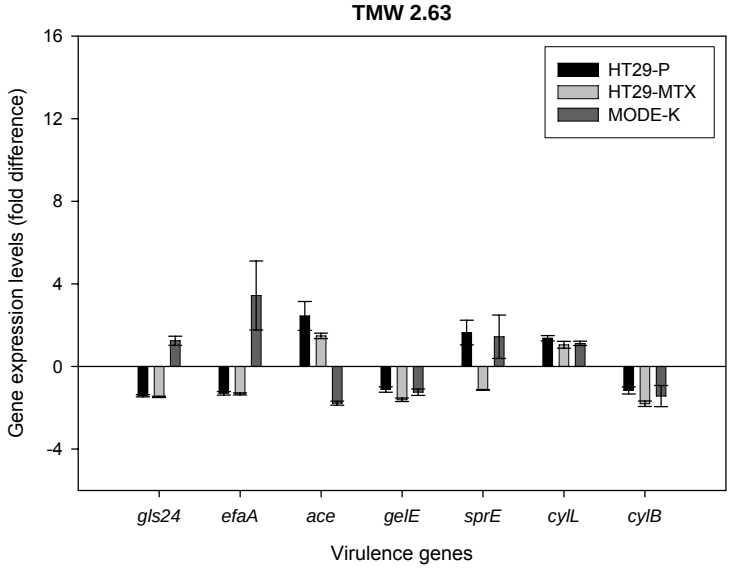


Figure 21. *Enterococcus faecalis* TMW 2.63 virulence gene expression levels in response to the presence of HT-29 P, HT-29 MTX and Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology.

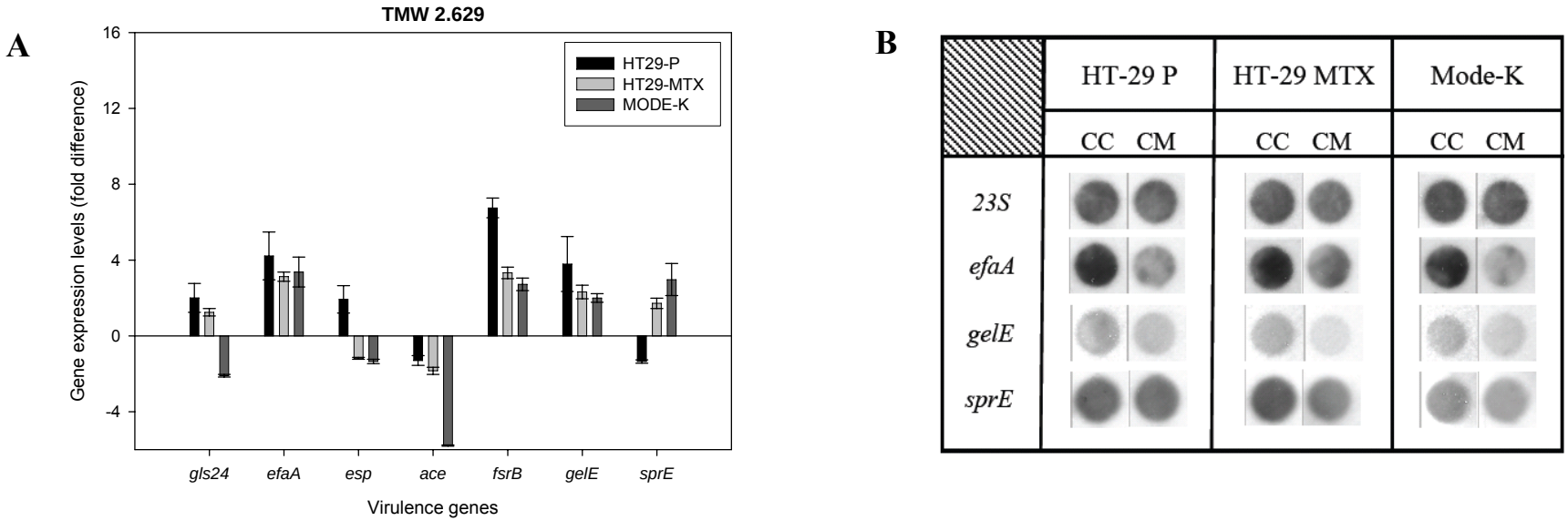


Figure 22. *Enterococcus faecalis* TMW 2.629 virulence gene expression levels in response to the presence of HT-29 P, HT-29 MTX and Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology (A) and by Northern (dot) blotting (B), where higher levels of expression are indicated by darker spots and gene expression levels in response to the presence of cell culture (CC) were compared to cell culture medium only (CM).

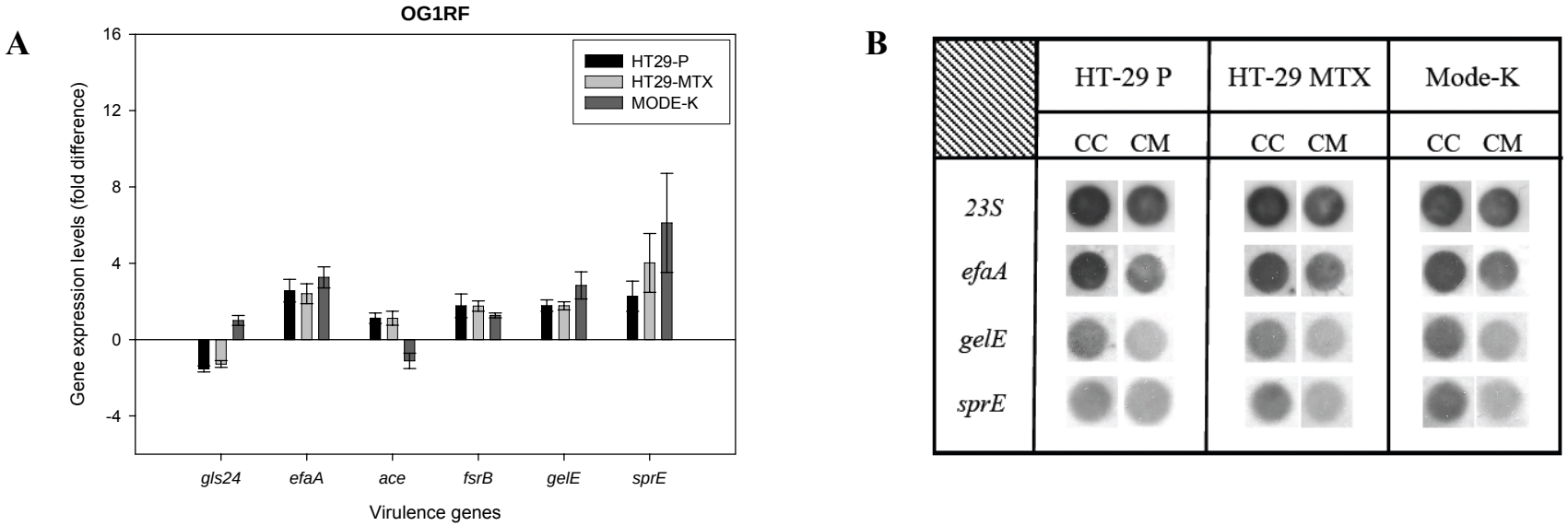


Figure 23. *Enterococcus faecalis* OG1RF virulence gene expression levels in response to the presence of HT-29 P, HT-29 MTX and Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology (A) and by Northern (dot) blotting (B), where higher levels of expression are indicated by darker spots and gene expression levels in response to the presence of cell culture (CC) were compared to cell culture medium only (CM).

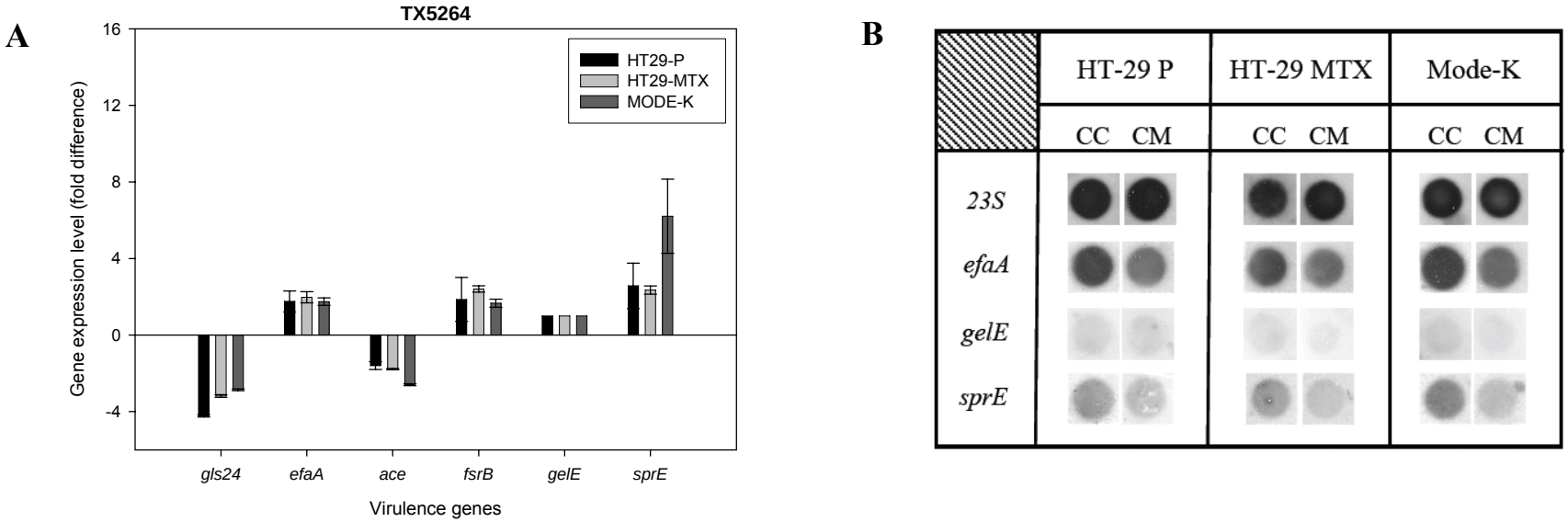


Figure 24. *Enterococcus faecalis* TX5264 virulence gene expression levels in response to the presence of HT-29 P, HT-29 MTX and Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology (A) and by Northern (dot) blotting (B), where higher levels of expression are indicated by darker spots and gene expression levels in response to the presence of cell culture (CC) were compared to cell culture medium only (CM).

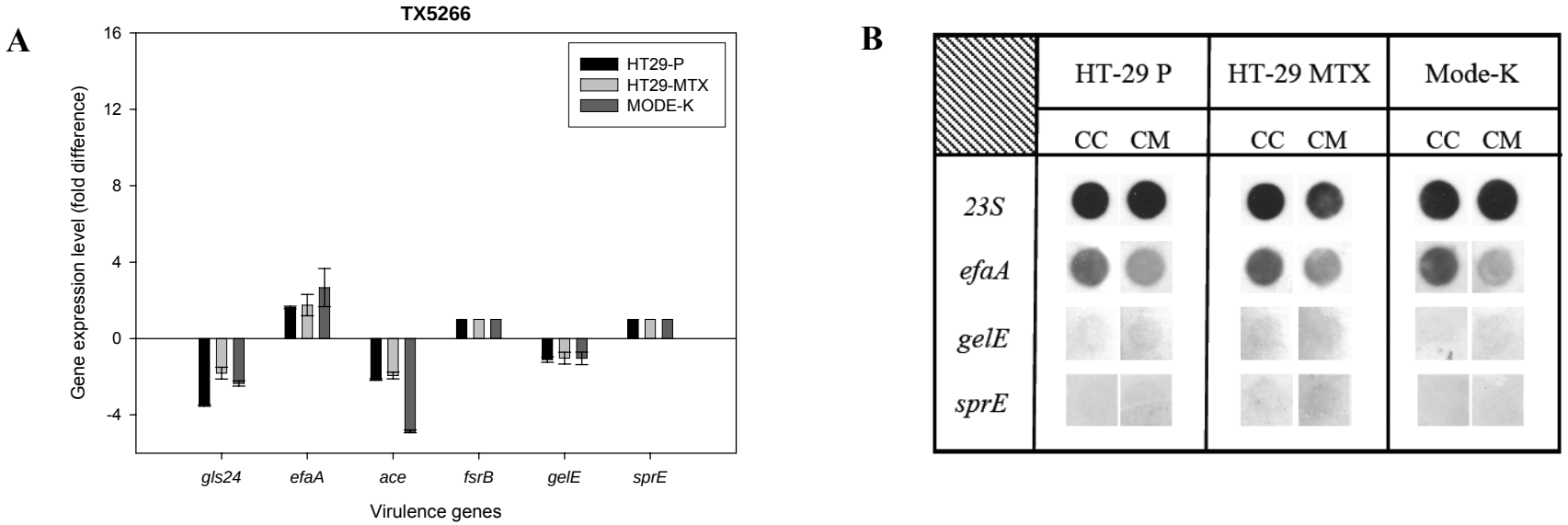
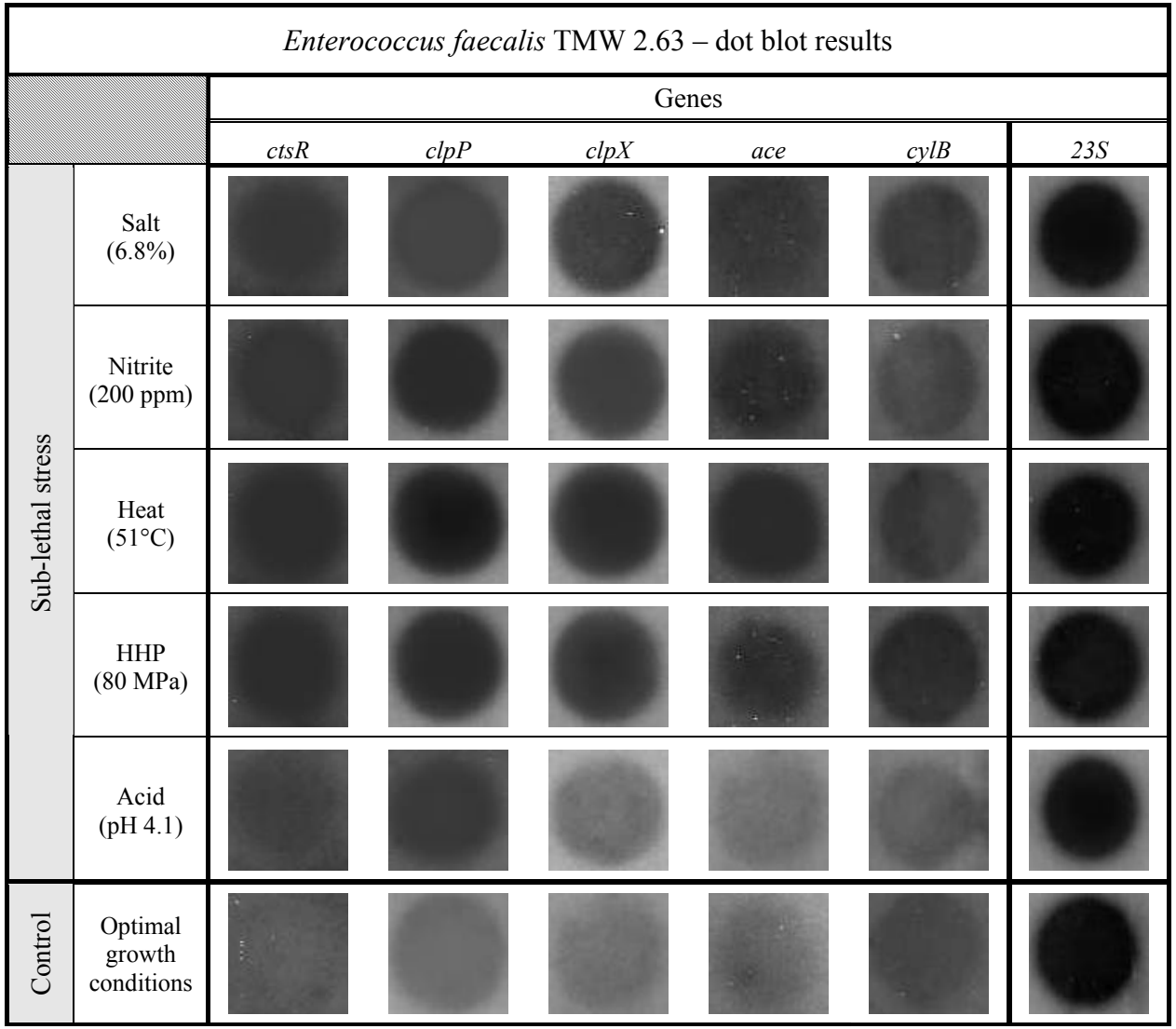


Figure 25. *Enterococcus faecalis* TX5266 virulence gene expression levels in response to the presence of HT-29 P, HT-29 MTX and Mode-K cells. mRNA levels were quantified by RT-PCR using Lightcycler® technology (A) and by Northern (dot) blotting (B), where higher levels of expression are indicated by darker spots and gene expression levels in response to the presence of cell culture (CC) were compared to cell culture medium only (CM).

Table 8. Northern (dot) blot analysis of *Enterococcus faecalis* TMW 2.63 virulence gene expression in response to sub-lethal stress. Stress conditions are listed in rows and genes examined are listed in columns. Spot intensity correlates with gene expression level, with darker spots indicating more gene expression and lighter spots indicating less gene expression. Gene expression levels in response to each stress were compared to optimal growth conditions (BHI broth with 0.5% NaCl, no nitrite, 37°C, 0.1 MPa, pH 7.2).



8 Curriculum Vitae

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9 Publications

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