

Research Article

Stress Effects on Virulence-related Genes Expression in *Enterococcus faecalis* from Food Source

^{1,2}Ming Lei, ¹Xianjun Dai and ¹Mingqi Liu

¹College of Life Sciences,

²Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, China Jiliang University, Hangzhou 310018, China

Abstract: *Enterococcus faecalis* are ubiquitous micro-organisms which are widely used in fermented food or probiotic industry. However, they are also associated with nosocomial infections and rank among the top three nosocomial bacterial pathogens. Some virulence factors were proposed which could strongly enhance their pathogenicity. In our study, three virulence-related genes *ace*, *gelE* and *efaA* were found in *E. faecalis* SL22 which was isolated from raw milk. The expression of these genes was examined in response to different stress factors like NaCl, pH and gentamycin sulfate by using real-time PCR. Significant differences in expression level were influenced by the environment factors, which suggests that these stress factors should be taken into consideration during the selection of enterococci for use in foods or as probiotics.

Keywords: *E. faecalis*, expression level, stress factor, virulence-related genes

INTRODUCTION

Enterococcus faecalis, belonging to the genus of *Enterococcus* known as Lactic Acid Bacteria (LAB), are Gram-positive, non-spore-forming, catalase-negative, oxidase-negative, facultative anaerobic cocci that occur singly, in pairs or in chains (Foulquié Moreno *et al.*, 2006; Stiles and Holzapfel, 1997). The *E. faecalis* are present in the microbial association of a variety of fermented foods, such as cheese, fermented sausages and vegetables. The bacteria have important implications in the food industry and play an acknowledged role in the development of organoleptic characteristics during the ripening of many cheeses or meats products.

Although enterococci have been successfully applied in food for a long time, in contrast to other LAB, these microorganisms are not Generally Recognized as Safe (GRAS) (Ogier and Serror, 2008). Currently, enterococci are among the most prevalent organisms encountered in hospital-associated infections, such as endocarditis; bacteremia; and urinary tract, Central Nervous System (CNS), intra-abdominal and pelvic infections (Sood *et al.*, 2008). The main enterococcal species involved in nosocomial infections are *E. faecalis* (80-90% of enterococcal isolates) (Miyazaki *et al.*, 2001). The differences between an enterococcal pathogen and an apparently safe food use strain are still unclear (Zago *et al.*, 2010). However, certain factors, such as Aggregation

substances (as), gelatinase (gel), cytolysin (cyl), enterococcal surface protein (esp), hyaluronidase (hyl), accessory colonization factor (ace) and endocarditis antigen (efa), have been implicated as potential virulence determinants (Eaton and Gasson, 2001; Martín-Platero *et al.*, 2009). These virulence determinants could play an important role in the pathogenicity of strains, including the colonisation and invasion of host tissues, as well as resistance towards specific and non-specific host defense mechanisms.

In this study, the investigation of the virulence-related genes presence and the expression levels of the detected genes in response to certain stress factors (NaCl, pH and gentamycin sulfate) were carried out. As the bacterial response to stress often coincides with increased virulence, a better understanding of how changes in these parameters affect gene expression in *E. faecalis* could provide helpful information for future risk assessment regarding the use of this organism in foods.

MATERIALS AND METHODS

Strains and growth conditions: An *E. faecalis* strain SL22 isolated from raw milk were kept in our laboratory and used in this study. The strain was identified by 16S rDNA sequencing analysis and stored at -80°C in MRS broth. To prepare cultures for subsequent stress experiments, the isolate were taken from glycerol stocks and grown overnight under

Corresponding Author: Xianjun Dai, College of Life Sciences, China Jiliang University, Xueyuan Street No. 258, Xiasha High Education Area, Hangzhou 310018, Zhejiang Province, P.R. China

This work is licensed under a Creative Commons Attribution 4.0 International License (URL: <http://creativecommons.org/licenses/by/4.0/>).

Table 1: PCR primers for the detection of virulence genes

Gene	Function	Primer sequences	Amplicon size (bp)	Reference
as	Adhesion	5'-AAGAAAAAAGTAACCAAC-3' 5'-AAACGGCAAGACAAGTAAATA-3'	1553	Domann <i>et al.</i> (2007)
cylA	Activation of cytolysin	5'-TGGATGATAGTGATAGGAAGT-3' 5'-TCT ACAGTAAATCTTTCGTCA-3'	517	Eaton and Gasson (2001)
esp	Colonization	5'-TTGCTAATGCTAGTCCACGAC-3' 5'-GCGTCAACACTTGCATTGCCGAA-3'	933	Eaton and Gasson (2001)
ace	Adhesion	5'-GGAATGACCGAGAACGATGGC-3' 5'-GCTTGATGTTGGCCTGCTTCCG-3'	616	Creti <i>et al.</i> (2004)
gelE	Tissue damage	5'-ACCCGTATCATTGGTTT-3' 5'-ACGCATTGCTTTTCCATC-3'	419	Eaton and Gasson (2001)
efaA	Colonisation	5'-ACCCGTATCATTGGTTT-3' 5'-ACGCATTGCTTTTCCATC-3'	688	Creti <i>et al.</i> (2004)
hyl	Tissue damage	5'-ACAGAAGCTGCAGGAAATG-3' 5'-GACTGACGTCCAAGTTTCCAA-3'	276	Vankerckhoven <i>et al.</i> (2004)

Table 2: Real-time PCR primers for the analysis of virulence genes expression

Gene	Primer sequences	Amplicon size (bp)
ace	5'-GGCGACTCAACGTTTGAC-3 5'-TCCAGCCAAATCGCCTAC-3	100
gelE	5'-GGAACAGACTGCCGGTTTAG-3 5'-TTCTGGATTAGATGCACCCG-3	103
efaA	5'-TGGGACAGACCCTCACGAATA-3 5'-CGCCTGTTTCTAAGTTCAAGCC-3	101
23S rRNA	5'-CCTATCGGCCTCGGCTTAG-3 5'-AGCGAAAGACAGGTGAGAATCC-3	101

anaerobic conditions at 37°C in MRS broth. Pre-warmed MRS medium was inoculated with 1% overnight cultures and grown an OD600 to 0.5 for the stress treatments.

Stress treatments: The method of Lenz *et al.* (2010) was used in the treatments and nutrient broth (Tryptone, 10 g/L and yeast extract 5 g/L) was used as base medium. The cell cultures (OD600 = 0.5) was divided and stress was induced either by gradient concentration of NaCl (0, 10, 20, 30 or 40 g/L, respectively), gradient pH value (pH 5.0, 6.0, 7.0, 8.0 or 9.0) and gentamycin sulfate (10 ng/mL), respectively. The stress conditions were applied for 3.0 h. Then the bacteria cells were centrifuged (10000 g; 10 min; 4°C) and the pellet with liquid nitrogen were stored at -80°C.

PCR screening for virulence-related genes: Genomic DNA was extracted from pure cultures using a Bacterial Genomic DNA Extraction Kit (Takara, Dalian, China) and PCR was used to detect the presence of the virulence determinants *as*, *cylA*, *esp*, *ace*, *hyl*, *gelE* and *efaA*. The primer sequences were synthesized by Sunny Biotechnology (Shanghai, China) and PCR procedures were set based related references (Table 1). The PCR products were then purified and ligated into pUCm T-vectors (Songon, Shanghai, China). The recombinant plasmids were transformed into *Escherichia coli* DH5α (Songon, Shanghai, China) and the inserted DNA was sequenced (Takara, Dalian, China). Database searches (BLAST programs, NCBI) were then applied to search for the sequence similarity.

RNA isolation and rewriting into cDNA: The bacteria pellet were treated with 3 mg/mL lysozyme (Songon, Shanghai, China) for 15 min, then total RNA was extracted using a Gram positive bacteria RNA extraction kit (Tiangen, Beijing, China) and treated with RNase-free DNaseI (Songon, Shanghai, China). The total RNA samples were stored at -80°C. Then the RNA (1 µg) were reverse transcribed into cDNA using a M-MuLV first strand cDNA synthesis kit according to the manufacturer's protocols (Songon, Shanghai, China). All the cDNA samples were stored at -20°C before use.

Real-time PCR analysis of virulence genes expression: The expression of harbored virulence related genes in the strain were analyzed under the stress environments. Real-time PCR and data analysis were performed by using the iCycler IQ real-time detection system (Bio-Rad, Hercules, CA) with SYBR Premix Ex Taq (Takara, Dalian, China). And comparative C_T (2^{-ΔΔCT}) method was used for the analysis of results. The real-time PCR primer sequences (Lenz *et al.*, 2010) were showed in Table 2 and synthesized by Sunny Biotechnology (Shanghai, China). Each PCR reaction contained SYBR Premix Ex Taq, 0.2 µL of each primer and 2 µM of a 1:10 dilution of the cDNA in a final volume of 20 µL. The real-time PCR programme was set as following: 94°C for 5 min and 40 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. All quantifications were normalized using 23s *rRNA* as an internal control.

Statistical analysis: The effect of the factors on virulence gene expression levels was investigated by three independent trials. Real-time PCR was conducted in duplicate for each trial. Data were analyzed using one-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Virulence determinants *as*, *cylA*, *esp*, *ace*, *hyl*, *gelE* and *efaA* were detected using PCR and DNA sequencing. The gene *ace*, *gelE* and *efaA* were found to harbor in the isolate (Fig. 1). The expression levels of the detected genes in response to the stress factors were

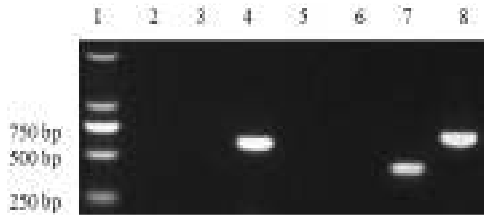


Fig. 1: Agarose gel electrophoresis of the PCR products of virulence factors for *E. faecalis* strain SL22

Lane 1: DNA marker; Lane 2: *as* gene; Lane 3: *cyla* gene; Lane 4: *ace* gene; Lane 5: *esp* gene; Lane 6: *hyl* gene; Lane 7: *gelE* gene; Lane 8: *efaA* gene; There were *ace*, *gelE* and *efaA* gene bands for the *E. faecalis* strain SL22

determined and are depicted in Fig. 2 to 4, for the factors of NaCl, pH and gentamycin sulfate,

respectively. The expression of *ace*, *gelE* and *efaA* were up-regulated when the concentration of NaCl increased, with the exception of the gene *efaA* at the concentration of 20 g/L NaCl. The expression levels of the *ace*, *gelE* and *efaA* were not significant different for the stress of pH from 5.0 to 8.0. When the pH value reached 9.0, expression levels of the three genes were significant up-regulated (for *ace*, 72.4-fold increasing; *gelE*, 9.8-fold increasing and *efaA*, 4.3-fold increasing). About the stress treatment of gentamycin sulfate, the expression levels of the three genes were different (for *ace*, 2.0-fold increasing; *gelE*, same level as blank and *efaA*, 0.3-fold decreasing). Overall, the expression levels of the three genes are up-regulated under most of the stress conditions (except for *efaA* under the stress of gentamycin sulfate).

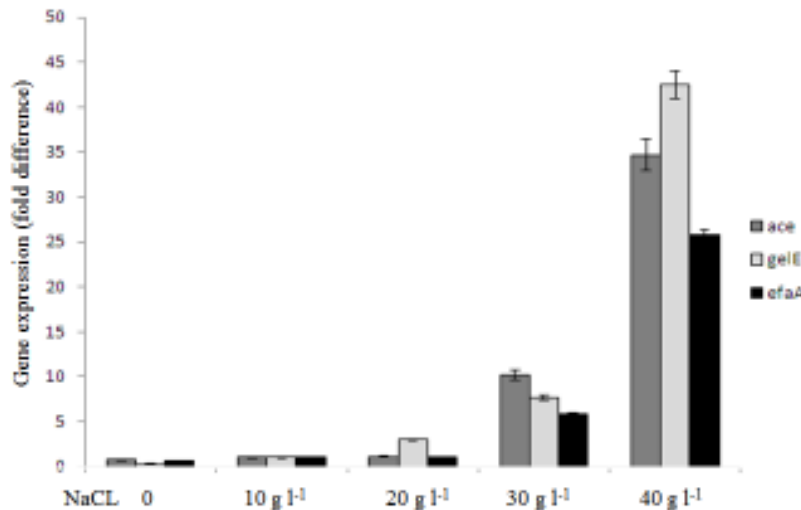


Fig. 2: The relative expression levels of virulence gene *ace*, *gelE* and *efaA* for *E. faecalis* strain SL22 after 3 h culture with different concentration of NaCl (0, 10, 20, 30 or 40 g/L)

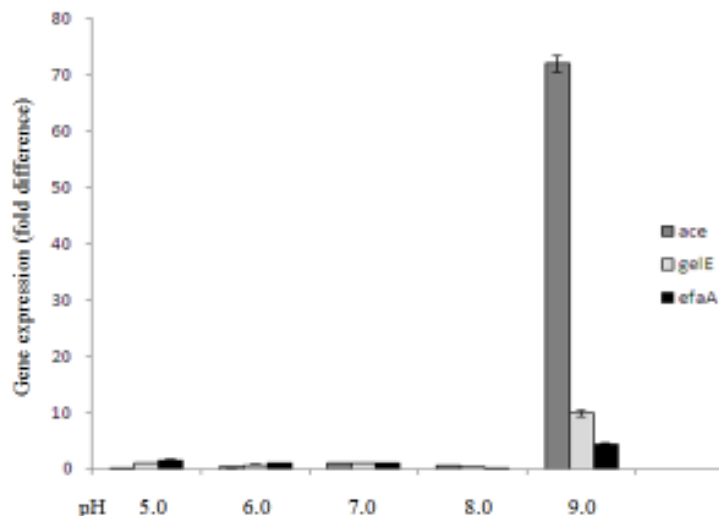


Fig. 3: The relative expression levels of virulence gene *ace*, *gelE* and *efaA* for *E. faecalis* strain SL22 after 3 h culture with different pH value (pH 5.0, 6.0, 7.0, 8.0 or 9.0)

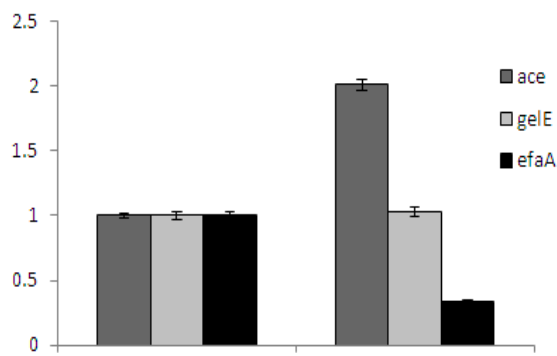


Fig. 4: The relative expression levels of virulence gene *ace*, *gelE* and *efaA* for *E. faecalis* strain SL22 after 3 h culture under the stress of gentamycin sulfate (10 ng/mL)

As is well-known, *E. faecalis*, a hardy organism, is able to survive harsh environmental conditions pertaining to temperature, salt, pH and some protease. Many of the chemical and physical stresses are very likely to appear in the environment of food processing or human gastrointestinal tract. There is a contradiction about the evaluation of *Enterococcus* to promote health or illness according to current information. As a controversial microorganism for food use, the previous studies focused on the identification of virulence factors and the impact on the pathogenicity of *E. faecalis*, little was known about environmental stresses and their contribution to the switch to their pathogenicity (Hew *et al.*, 2007; Lenz *et al.*, 2010). Based on a large number of literature reports, the change of certain environmental factors could significantly influence the expression levels of virulence genes and the increasing expression may strongly enhance the pathogenicity of pathogens. In the present study, the applied environmental stress factors are likely to appear in food processing or probiotics intake and the identification of such responses demonstrates the strain SL22 has the capability of sensing the environment change and regulating the related virulence genes expression in response, which imply the *E. faecalis* are not completely safe.

CONCLUSION

In this study, the virulence determinants *ace*, *gelE* and *efaA* were found in *E. faecalis* strain SL22 and the strain could sense the environmental change (stress factors of NaCl, pH and gentamycin) and regulate the expression levels of virulence genes (most are up-regulated). For the consumer's safety, these stress factors should be taken into consideration during the selection of enterococci for use in foods or as probiotics.

ACKNOWLEDGMENT

This research was financially supported by funding (2012C13SA190002) from the Science Technology Department of Zhejiang Province, China.

REFERENCES

- Creti, R., M. Imperi, L. Bertuccini, F. Fabretti, G. Orefici, R. Di Rosa and L. Baldassarri, 2004. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J. Med. Microbiol.*, 53: 13-20.
- Domann, E., T. Hain, R. Ghai, A. Billion, C. Kuenne, K. Zimmermann and T. Chakraborty, 2007. Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic *Enterococcus faecalis* strain Symbioflor 1. *Int. J. Med. Microbiol.*, 297: 533-539.
- Eaton, T.J. and M.J. Gasson, 2001. Molecular screening of enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microb.*, 67: 1628-1635.
- Foulquié Moreno, M.R., P. Sarantinopoulos, E. Tsakalidou and L. De Vuyst, 2006. The role and application of enterococci in food and health. *Int. J. Food. Microbiol.*, 106(1): 1-24.
- Hew, C.M., M. Korakli and R.F. Vogel, 2007. Expression of virulence-related genes by *Enterococcus faecalis* in response to different environments. *Syst. Appl. Microbiol.*, 30(4): 257-267.
- Lenz, C.A., C.M. Hew Ferstl and R.F. Vogel, 2010. Sub-lethal stress effects on virulence gene expression in *Enterococcus faecalis*. *Food Microbiol.*, 27(3): 317-326.
- Martín-Platero, A.M., E. Valdivia, M. Maqueda and M. Martínez-Bueno, 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *Int. J. Food Microbiol.*, 132: 24-32.
- Miyazaki, S., T. Fujikawa, I. Kobayashi, T. Matsumoto, K. Tateda and K. Yamaguchi, 2001. Development of systemic bacteraemia after oral inoculation of vancomycin-resistant enterococci in mice. *J. Med. Microbiol.*, 50: 695-701.
- Ogier, J.C. and P. Serror, 2008. Safety assessment of dairy microorganisms: The *Enterococcus* genus. *Int. J. Food Microbiol.*, 126: 291-301.
- Sood, S., M. Malhotra, B. Das and A. Kapil, 2008. Enterococcal infections and antimicrobial resistance. *Indian J. Med. Res.*, 128: 111-121.
- Stiles, M.E. and W.H. Holzapfel, 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.*, 36: 1-29.

- Vankerckhoven, V., T. Van Autgaerden, C. Vael, C. Lammens, S. Chapelle, R. Rossi, D. Jabes and H. Goossens, 2004. Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp* and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J. Clin. Microbiol.*, 42: 4473-4479.
- Zago, M., G. Huys and G. Giraffa, 2010. Molecular basis and transferability of tetracycline resistance in *Enterococcus italicus* LMG 22195 from fermented milk. *Int. J. Food Microbiol.*, 142: 234-236.