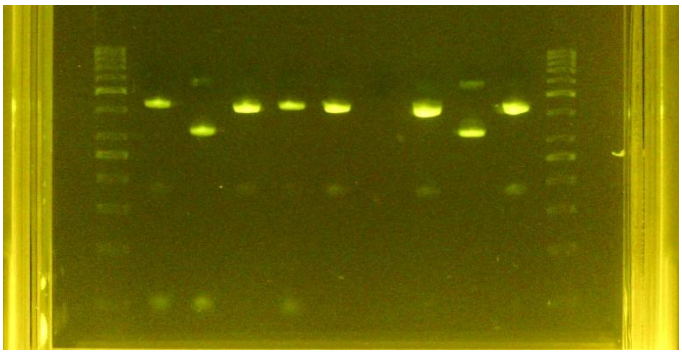
LABORATORY PROTOCOL

PCR-based differentiation and identification of *Enterococcus faecium* and *Enterococcus lactis*



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Background

Harmonised monitoring of antimicrobial resistance in the EU has included the two main human enterococcal pathogens *Enterococcus faecalis* and *Enterococcus faecium*, with Member States (MSs) voluntarily reporting relevant findings to the European authorities (1).

Nevertheless, it was recently proposed that *E. faecium* clade B is phylogenetically and clinically distinct from *E. faecium* clade A (2,3). To reflect that, clade B has now been reclassified as *Enterococcus lactis*. However, methodologies for the identification of *E. faecium*, which were developed prior to this reclassification, cannot reliably distinguish between *E. faecium* and *E. lactis* isolates. As a result, isolates previously identified as *E. faecium* within surveillance systems may, in fact, belong to *E. lactis*.

Accurate identification and differentiation of enterococcal isolates are essential for surveillance purposes, particularly in light of evidence suggesting that the pathogenicity and antimicrobial resistance profiles of *E. lactis* may differ from those of *E. faecium* (2,3).

The present protocol is intended for the identification and differentiation of *E. faecium* and *E. lactis* isolates using either a multiplex PCR amplification or two separate PCR reactions, one for identification of *E. faecium* and a second for identification of *E. lactis*. The PCR methodology is based on a published method developed by Belloso Daza *et al*. (2) and relies on the amplification of species-specific fragments of the *gluP* gene, which encodes a rhomboid protease.

In their methodological paper (2), the authors found this method to allow species identification with 100 % specificity and no cross-reactivity. However, despite the high specificity of the *gluP* gene for differentiating *E. faecium* and *E. lactis*, a subsequent large-scale *in silico* analysis of whole-genome sequences available in public repositories identified rare cases in which *E. faecium* strains of clades A1 and A2 carried *gluP* gene sequences that appeared to be hybrids between *E. faecium* and *E. lactis* (4). Based on *in silico* predictions, these rare isolates would not be distinguishable using the *gluP* PCR differentiation method (4). As these genomes have only been found online and the findings are based solely on *in silico* analyses, it remains unclear whether these strains genuinely harbour mosaic *gluP* sequences derived from both species or whether the observed discrepancies result from sequencing, assembly, or contamination artifacts. Nevertheless, this possibility should be taken into account when interpreting the PCR results. Further investigation is warranted to determine whether such strains truly exist and, if so, to establish their prevalence and significance.

In the present protocol we have adapted the two-step differentiation method proposed by Belloso Daza *et al*. (2) into a single-step multiplex PCR, which can be used by laboratories for rapid screening of isolates suspected to be *E. faecium* or *E. lactis*, e.g. following their identification as *E. faecium*/*E. lactis* in the multiplex PCR method proposed by Dutka-Malen (5), or the respective EURL-AMR protocol ("Protocol for PCR amplification of *E. faecium* and *E. faecalis*" available at https://www.food.dtu.dk/english/topics/antimicrobial-resistance/eurl-ar/protocols). The multiplex PCR proposed here is easy-to-use and, when tested on 50 historic isolates of the EURL-AMR's culture collection, could easily discriminate between the two species. However, it should be noted that with this method, when visualizing the PCR products, besides the strong bands of the species-specific amplicons, weak unspecific bands can also be seen. This does not deter from the easy identification of the isolates (Figure 1) and we, thus, do not believe that it is a hindering factor for using this method for rapid isolate screening. Nonetheless, we also provide here an alternative protocol for the two separate PCR reactions for the identification of *E. faecium* and *E. lactis*, as originally proposed by Belloso Daza *et al*., which reduces the background in the amplification and gel. It is up to each laboratory to decide which of the two methodologies to use.

This protocol details the two PCR procedures for differentiation and identification of *E. faecium* and *E. lactis* step-by-step and includes explanations on the theory behind each step. It can be used in tandem with the "Protocol for PCR amplification of *E. faecium* and *E. faecalis*", which can be found on the EURL-AMR website (https://www.food.dtu.dk/english/topics/antimicrobial-resistance/eurl-ar/protocols).

Procedure		Theory/comments
1. DNA extraction (boiling lysates)		
1.1. Using a sterile 1 µL inoculation loop, pick a single colony of the isolate of interest (approximately one loopful of biomass) and suspend it in 1 mL of Milli-Q water in a 1.5 mL microcentrifuge (Eppendorf) tube.		Both at this step as well as in steps 1.3 and 1.7, it is possible instead of Milli-Q water to use any other equivalent-grade purified water.
1.2. Centrifuge the tube at 20,000 g for 5 min.		
1.3. Carefully remove the supernatant using a pipette and resuspend the pellet in 100 µL of Milli-Q water.		
1.4. Place the Eppendorf tube in a preheated heating block and incubate at 100 °C for 10 min.		
1.5. Centrifuge the tube at 20,000 g for 5 min.		The centrifugation speed proposed here is indicative, and can be adjusted to the maximum speed supported by the individual centrifuge.
1.6. Using a pipette, transfer the supernatant to a new tube.		Ensure that the pipette tip does not touch the pellet.
1.7. Dilute the sample 1:10 in Milli-Q water.		This sample will be used as the DNA template for the PCR reaction(s).
1.8. Store the sample at -20 °C.		

2. Multiplex PCR for differentiation and identification of *E. lactis* and *E. faecium*

2.1. Preparation of PCR primers

Prepare a stock solution of each primer of Table S1, Appendix 1, at a concentration of 10 µM.

The multiplex PCR reaction mix will include 0.5 µL of the stock solution of each primer, corresponding to approximately 0.2 µM of each primer in the final PCR reaction.

2.2. Preparation of the multiplex PCR reaction mix

2.2.1. Prepare a PCR reaction mastermix sufficient to cover the number of samples tested plus one additional negative-control reaction, plus two additional positive-control reactions, plus approximately 10% extra reactions to account for pipetting inaccuracies.

Below is the composition of the mastermix from the protocol used at the EURL-AMR, which has been optimized for the DreamTaq Green PCR mix. The amount for 5 reactions should be sufficient to test 1 sample, including the positive and negative controls.

Component	**1 reaction (µL)**	**5 reactions (µL)**
DreamTaq Green PCR Mastermix 2x	12.5	62.5
FW primer Efm_GluP_F	0.5	2.5
REV primer Efm_GluP_R	0.5	2.5
FW primer El_GluP_F	0.5	2.5
REV primer El_GluP_R	0.5	2.5
Nuclease-free water	5.5	27.5
Total volume	**20**	**100**

The amounts and conditions shown here should be adjusted based on the reagents and conditions employed by the individual laboratory.

It is possible to replace the DreamTaq polymerase with another polymerase, according to the laboratory's normal procedures.

If the composition of the mastermix is to be adjusted to the polymerase used by the individual laboratory, it should be taken into consideration that the DreamTaq Green PCR Mastermix 2x contains dNTP and $MgCl_2$, in amounts corresponding to 0.2 mM of each dNTP and 2 mM $MgCl_2$ in the final PCR reaction.

In addition, it should be noted that the DreamTaq Green PCR Mastermix 2x includes a density reagent and two dyes. This allows direct loading of the PCR reaction on an agarose gel and monitoring of the electrophoresis without the necessity for staining or additional dyes.

2.2.2. Pipet 20 µL of PCR mastermix into each microcentrifuge tube. The number of tubes should correspond to the number of samples to be tested plus one additional negative-control reaction, plus one positive control for *E. lactis*, plus one positive control for *E. faecium*.		
2.2.3. Label the tubes and add the following templates to the corresponding tube: • 5 µL of DNA from a known *E. lactis* strain (positive control for *E. lactis*) • 5 µL of DNA from a known *E. faecium* strain (positive control for *E. faecium*) • 5 µL of test isolate DNA (step 1.7) • 5 µL of nuclease-free water (negative control)		Each reaction mixture (25 µl) comprises the following: • 12.5 µL DreamTaq Green PCR Mastermix 2x (corresponds to 0.2 mM of each dNTP and 2 mM $MgCl_2$ in the final reaction) • 0.2 µM of each primer (0.5 µL of stock solution; see step 2.1) • Template DNA (5 µL of DNA sample from steps 1.7-1.8) • 5.5 µl nuclease-free water
2.3. Run multiplex PCR reaction		
2.3.1. Run the multiplex PCR reaction for identification of *E. lactis* and *E. faecium* at the following conditions: 1. Initial denaturation: 10 min at 94 °C 2. 30 cycles of: i. 30 s at 94 °C (denaturation) ii. 30 s at 60 °C (annealing) iii. 30 s at 72 °C (extension) 3. Final extension: 10 min at 72 °C 4. Hold at 4 °C		

2.4. Electrophoresis		
2.4.1. On a 2% agarose gel, load: • Lane 1: A 50 bp ladder as a molecular weight marker. • Lane 2: 4 µL of amplified *E. lactis* DNA (positive control). • Lane 3: 4 µL of amplified *E. faecium* DNA (positive control). • Lane 3: 4 µL negative control (Mastermix without template). • Lane 4-…: 4 µL of the PCR product from each sample.		The loading volume may be adjusted, depending on the efficiency of the PCR and the intensity of the band when visualized on the gel. If the PCR buffer used is not suitable for direct loading on the gel, add loading buffer to each PCR reaction before loading. If the PCR buffer used does not include dyes for direct visualization, staining of the gel with ethidium bromide or another dye may be required.
2.4.2. Run until bands are clearly separated.		The expected amplicons differ by only 103 bp. To achieve clear band separation, it may be necessary to extend the gel running time and/or reduce the current.
2.4.3. Visualize the gel. Observe the bands and interpret the results according to Figure 1.		Ensure that there are the expected bands for the two positive controls and no band for the negative control.

3. Alternative method: 2-step PCR-based identification of *E. lactis* and *E. faecium*

This is the same methodology as for the multiplex PCR but split in two separate PCR reactions instead. The advantage in this case is that the PCR reactions are more specific, which reduces the background amplicons.

3.1. Preparation of PCR primers

Prepare a stock solution of each primer of Table S1, Appendix 1, at a concentration of 10 µM.

Each PCR reaction will include 0.5 µL of the stock solution of each corresponding primer, equivalent to approximately 0.2 µM of each primer in the final PCR reaction.

3.2. Preparation of PCR reaction mixes

Prepare a PCR reaction mastermix for the *E. lactis* PCR and a PCR reaction mastermix for the *E. faecium* PCR, each sufficient to cover the number of samples tested plus one additional negative-control reaction, plus two additional positive control reactions, plus approximately 10% extra reactions to account for pipetting inaccuracies.

The following sections describe the composition of the mastermixes based on the protocol used at the EURL-AMR, which has been optimized for the DreamTaq Green PCR mix. The amount for 5 reactions should be sufficient to test 1 sample, including the positive and negative controls

The amounts and conditions shown here should be adjusted based on the reagents and conditions employed by the individual laboratory.

It is possible to replace the DreamTaq polymerase with another polymerase, according to the laboratory's normal procedures.

If the composition of the mastermix is to be adjusted to the polymerase used by the individual laboratory, it should be taken into consideration that the DreamTaq Green PCR Mastermix 2x contains dNTP and $MgCl_2$, in amounts corresponding to 0.2 mM of each dNTP and 2 mM $MgCl_2$ in the final PCR reaction.

In addition, it should be noted that the DreamTaq Green PCR Mastermix 2x includes a density reagent and two dyes. This allows direct loading of the PCR reaction on an agarose gel and monitoring of the electrophoresis without the necessity for staining or additional dyes.

***E. lactis* PCR**

3.2.1. Prepare the reaction mastermix for the *E. lactis* PCR.

Component	**1 reaction (µL)**	**5 reactions (µL)**
DreamTaq Green PCR Mastermix 2x	12.5	62.5
FW primer El_GluP_F	0.5	2.5

REV primer El_GluP_R	0.5	2.5
Nuclease-free water	6.5	32.5
Total volume	**20**	**100**

3.2.2. Pipet 20 µL of PCR mastermix into each microcentrifuge tube. The number of tubes should correspond to the number of samples to be tested plus one additional negative-control reaction, plus one positive control with a known *E. lactis*, plus one negative control with a known *E. faecium* strain.

3.2.3. Label the tubes and add the following templates to the corresponding tube:

- 5 µL of DNA from a known *E. lactis* strain (positive control for *E. lactis*)
- 5 µL of DNA from a known *E. faecium* strain (negative control for *E. faecium*)
- 5 µL of test isolate DNA (step 1.7)
- 5 µL of nuclease-free water (negative control)

Each reaction mixture (25 µl) comprises the following:

- 12.5 µL DreamTaq Green PCR Mastermix 2x (corresponds to 0.2 mM of each dNTP and 2 mM $MgCl_2$ in the final reaction)
- 0.2 µM of each primer (0.5 µL of stock solution; see step 2.1)
- Template DNA (5 µL of DNA sample from steps 1.7-1.8)
- 6.5 µl nuclease-free water

***E. faecium* PCR**

3.2.4. Prepare the reaction mastermix for the *E. faecium* PCR.

Component	**1 reaction (µL)**	**5 reactions (µL)**
DreamTaq Green PCR Mastermix 2x	12.5	62.5
FW primer Efm_GluP_F	0.5	2.5
REV primer Efm_GluP_R	0.5	2.5
Nuclease-free water	6.5	32.5
Total volume	**20**	**100**

3.2.5. Pipet 20 µL of PCR mastermix into each microcentrifuge tube. The number of tubes should correspond to the number of samples to be tested plus one additional negative-control reaction, plus one positive control with a known *E. faecium*, plus one negative control with a known *E. lactis* strain.		
3.2.6. Label the tubes and add the following templates to the corresponding tube: • 5 µL of DNA from a known *E. lactis* strain (negative control for *E. lactis*) • 5 µL of DNA from a known *E. faecium* strain (positive control for *E. faecium*) • 5 µL of test isolate DNA (step 1.7) • 5 µL of nuclease-free water (negative control)		Each reaction mixture (25 µl) comprises the following: • 12.5 µL DreamTaq Green PCR Mastermix 2x (corresponds to 0.2 mM of each dNTP and 2 mM $MgCl_2$ in the final reaction) • 0.2 µM of each primer (0.5 µL of stock solution; see step 2.1) • Template DNA (5 µL of DNA sample from steps 1.7-1.8) • 6.5 µl nuclease-free water
3.3. Run the PCRs		
***E. lactis* PCR**		
3.3.1. Run the PCR reactions for the *E. lactis* PCR at the following conditions: • Initial denaturation: 10 min at 94 °C • 30 cycles of: i. 30 s at 94 °C (denaturation) ii. 30 s at 58 °C (annealing) iii. 30 s at 72 °C (extension) • Final extension: 10 min at 72 °C • Hold at 4 °C		
***E. faecium* PCR**		
3.3.2. Run the PCR reactions for the *E. faecium* PCR at the following conditions: • Initial denaturation: 10 min at 94 °C		

• 30 cycles of: i. 30 s at 94 °C (denaturation) ii. 30 s at 61 °C (annealing) iii. 30 s at 72 °C (extension) • Final extension: 10 min at 72 °C • Hold at 4 °C		
3.4. Electrophoresis		
***E. lactis* PCR**		
3.4.1. On a 2% agarose gel, load: • Lane 1: A 50 bp ladder as a molecular weight marker. • Lane 2: 4 µL of amplified *E. lactis* DNA (positive control). • Lane 3: 4 µL of amplified *E. faecium* DNA (negative control). • Lane 3: 4 µL negative control (*E. lactis* PCR Mastermix without template). • Lane 4-…: 4 µL of the PCR product from each sample.		The loading volume may be adjusted, depending on the efficiency of the PCR and the intensity of the band when visualized on the gel. If the PCR buffer used is not suitable for direct loading on the gel, add loading buffer to each PCR reaction before loading. If the PCR buffer used does not include dyes for direct visualization, staining of the gel with ethidium bromide or another dye may be required.
3.4.2. Run until bands are clearly separated.		
3.4.3. Visualize the gel. Observe the bands and interpret the results according to Figure 1.		Ensure that there is the expected band for the positive control and no band for the two negative controls.
***E. faecium* PCR**		
3.4.4. On a 2% agarose gel, load: • Lane 1: A 50 bp ladder as a molecular weight marker. • Lane 2: 4 µL of amplified *E. lactis* DNA (negative control). • Lane 3: 4 µL of amplified *E. faecium* DNA (positive control).		The loading amounts may be adjusted, depending on the efficiency of the PCR and the intensity of the band when visualized on the gel. If the PCR buffer used is not suitable for direct loading on the gel, add loading buffer to each PCR reaction before loading. If the PCR buffer used does not include dyes for direct visualization, staining of the gel with

• Lane 3: 4 µL negative control (*E. lactis* PCR Mastermix without template). • Lane 4-…: 4 µL of the PCR product from each sample.		ethidium bromide or another dye may be required.
3.4.5. Run until bands are clearly separated.		
3.4.6. Visualize the gel. Observe the bands and interpret the results according to Figure 1.		Ensure that there is the expected band for the positive control and no band for the two negative controls.

Figures

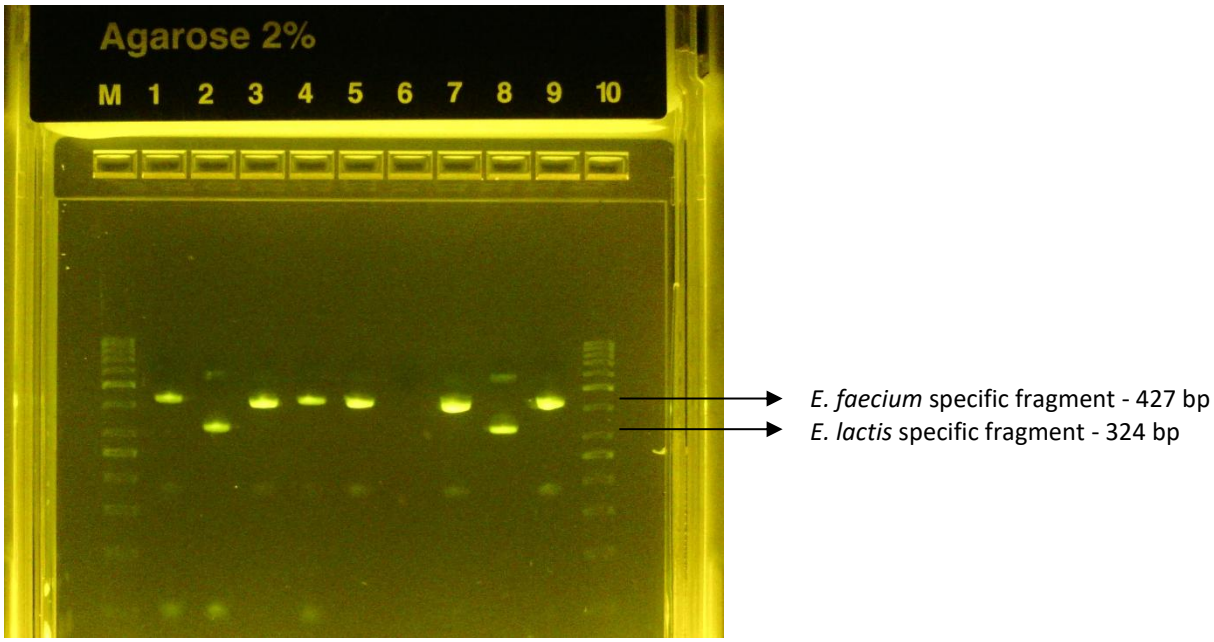

Figure 1. Visualization of multiplex PCR reactions for detection of *E. faecium and E. lactis* on a 2% agarose e-gel, flanked by the 50 bp GeneRuler ladder. Isolates in lanes 1, 3-5, 7 and 9 are identified as *E. faecium*, whereas isolates in lates 2 and 8 as *E. lactis*.

References

(1) Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria and repealing Implementing Decision 2013/652/EU.

(2) Belloso Daza MV, Almeida-Santos AC, Novais C, Read A, Alves V, Cocconcelli PS, Freitas AR, Peixe L. Distinction between *Enterococcus faecium* and *Enterococcus lactis* by a *gluP* PCR-based assay for accurate identification and diagnostics. Microbiol Spectr. 2022 Dec 21;10(6):e0326822.

(3) Belloso Daza MV, Cortimiglia C, Bassi D, Cocconcelli PS. 2021. Genome-based studies indicate that the *Enterococcus faecium* clade B strains belong to *Enterococcus lactis* species and lack of the hospital infection associated markers. Int J Syst Evol Microbiol 71:004948.

(4) Tedim AP, Almeida-Santos AC, Lanza VF, Novais C, Coque TM, Freitas AR, Peixe L; from the ESCMID Study Group on Food- and Water-borne Infections (EFWISG). Bacteriocin distribution patterns in *Enterococcus faecium* and *Enterococcus lactis*: bioinformatic analysis using a tailored genomics framework. Appl Environ Microbiol. 2024 Oct 23;90(10):e0137624.

(5) Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995 Jan;33(1):24-7.

APPENDIX 1

Table S1. Primers for identification of *E. lactis* and *E. faecalis* via PCR

Target gene	Primer name (# EURL)	Sequence (5' to 3')	Amplicon size	Targeted species
gluP	Efm_GluP_F	GCGTGCATGGTTAAGACGAC	427 bp	*E. faecium*
	Efm_GluP_R	CTGCTGGATCGCTGGGTTAT		
	El_GluP_F	TACGGTCACTGGCGGTTTTT	324 bp	*E. lactis*
	El_GluP_R	TGTCTGCTGTTTCGGTAGCC		