



LABORATORY PROTOCOL

Isolation of *Enterococcus faecalis* and *Enterococcus faecium* from mussels

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**April 2026
Version 1**

HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
1	New document	First draft of the SOP	April 2026	

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Background

To harmonise the antimicrobial resistance (AMR) monitoring systems in the European Union (EU), the European Commission (EC) has adopted legislation (1) laying down detailed rules for the monitoring and reporting of AMR in zoonotic and commensal bacteria by Member States (MSs). The legislation provides a framework for routine monitoring of AMR in bovine animals, pigs and poultry. Complementary to that, the European Food Safety Authority (EFSA) has additionally recommended to undertake baseline surveys (BLSs) to assess the epidemiological situation on specific AMR issues (2). One such highlighted topic is the assessment of the prevalence of AMR in bacteria from aquaculture animals. This is of importance, as, at present, there are only limited data on the occurrence of AMR in aquaculture production in Europe and the data that are available cannot easily be compared due to methodological differences.

To address this need, in accordance with Article 31 of Regulation (EC) No 178/2002, EFSA was requested to provide technical and scientific support for the development of a BLS on the prevalence of AMR in bacteria isolated from EU-produced aquaculture animals, including proposed harmonised approaches for the collection and the analysis of AMR data from aquaculture animals (3). Target organisms selected for the BLS include *Enterococcus faecalis* and *Enterococcus faecium* (3).

Similar to commensal *Escherichia coli*, enterococci can serve as indicators of faecal contamination and anthropogenic pollution in aquatic environments and aquaculture products. Moreover, it has been proposed that enterococci may also be relevant indicators for monitoring the levels of AMR at aquaculture production sites (3). However, their use as AMR indicators has so far been limited by the lack of robust and comparable literature on the occurrence of AMR among *Enterococcus* species. (3). The inclusion of enterococci as target organisms in the BLSs is expected to generate harmonised and comparable data on the occurrence of *Enterococcus*-borne AMR in aquaculture products and thereby inform risk assessment (3). In addition, it is expected to complement the BSL data for the Gram-negative *E. coli*, with data from a Gram-positive indicator organism which is not influenced by the pressures imposed by AMR-targeted control measures (3).

In terms of species selection, although various *Enterococcus* species have been detected in aquaculture products, for the BLSs it was selected to focus on the main human pathogens *E. faecalis* and *E. faecium*, as these are the most relevant species for AMR monitoring from a public health perspective (3).

The present protocol forms part of a series of protocols that together with the “Technical specifications for a EU-wide baseline survey of antimicrobial resistance in bacteria from aquaculture animals” (3) aim to provide harmonised methods for AMR monitoring of bacteria in aquaculture productions, including BLSs. Specifically, the protocol is intended for use by MSs for the isolation of *E. faecalis* and *E. faecium* from samples of mussels.

The protocol details the procedure step-by-step and includes explanations on the theory behind each step. It can be used in tandem with the following protocols:

- Overview of sampling, pre-enrichment and laboratory analysis of samples for aquaculture baseline surveys
- Identification of bacteria with MALDI-TOF (Matrix-Assisted Laser Desorption-Ionization Time Of Flight)
- Susceptibility testing by MIC of bacteria from aquaculture animals
- Isolation of *Escherichia coli* and *Klebsiella pneumoniae* from mussels
- Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from mussels
- PCR-based identification of *Enterococcus faecalis* and *Enterococcus faecium*

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Procedure	Theory/comments
<p>1. Isolation of <i>E. faecalis</i> and <i>E. faecium</i></p>	<p>The procedure is outlined in the flowchart of Appendix 2.</p>
<p>1.1. Samples should be transported to the laboratory at a temperature between 0 and 8 °C. Samples should arrive at the laboratory and be initially processed within 24 hours of sampling, where possible. The maximum acceptable time for initial processing is 72 hours from the time of sampling.</p> <p>Upon receiving the samples at the laboratory, inspect them visually and discard any samples with damaged packaging or incorrect labelling. Discard any samples that did not arrive to the laboratory within 72 hours of sampling as well as any samples which have not been kept at the appropriate temperature during transport or storage.</p>	<p>For instructions on sampling, refer to section 6.2 of the document “Technical specifications for a baseline survey on AMR in aquaculture animals” (3). Please note that the baseline study will focus on samples from healthy live mussels.</p> <p>It is necessary to keep the samples at a temperature approaching that of melting ice to avoid unreliable results. Samples should not be frozen.</p> <p>During transport and storage prior to analysis, samples should be handled according to the ISO 7218 standard: “Microbiology of the food chain – General requirements and guidance for microbiological examinations”.</p> <p>For further details, refer to the Guidance document on official controls under Regulation (EU) 2017/625 (4), and the Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 that lays down specific hygiene rules for food of animal origin (5).</p>
<p>1.2. Store samples at the laboratory between 0 and 8 °C until microbiological analysis. Analyse samples within 72 h of sampling, preferably immediately after samples are received and within 24 hours of sampling. It must be ensured that the cold chain is maintained at all times between sample collection and analysis.</p>	<p>For storage prior to analysis, samples should be handled in accordance with the ISO 7218 standard: “Microbiology of the food chain – General requirements and guidance for microbiological examinations”.</p> <p>Samples should not be frozen.</p>
<p>1.3. Pre-enrichment: Add 25 ± 0.5 g of mussel sample to 225 mL of buffered peptone water (BPW, Appendix 1) and homogenise.</p>	<p>Homogenization may be performed using a peristaltic blender or other comparable methods.</p>
<p>1.4. Incubate the pre-enrichment culture in appropriate sterile tubes/beakers with lids at 37 ± 1 °C for 18-22 h. Alternatively, sterile Stomacher bags can also be used for incubation, if this is in accordance with the laboratory’s procedures.</p>	<p>To minimize the risk of spillage, it is recommended to avoid shaking the tubes/containers.</p> <p>Note: The pre-enrichment culture can be used in parallel for the isolation of other indicator bacteria included in the BSL, such as commensal and ESBL/AmpC/carbapenemase-producing <i>E. coli</i> and <i>K. pneumoniae</i>. For an overview of how to consolidate this step, please refer to the protocol “Overview of sampling, pre-enrichment and</p>

Procedure	Theory/comments
	laboratory analysis of samples for aquaculture baseline surveys".
<p>1.5. After mixing gently the incubated pre-enrichment culture in BPW (step 1.4), subculture one loopful (10 µL inoculation loop) by applying a single streak onto a Slanetz and Bartley agar plate. From this streak, make two additional streaks sequentially, using either the same loop or a new loop, to ensure growth of single colonies. Incubate the plates at 37 ± 1 °C for 48 h under aerobic conditions.</p>	<p>Slanetz and Bartley agar plates are commercially available.</p> <p>Alternatively, details on the composition and preparation of Slanetz and Bartley agar plates, together with safety considerations, can be found in ISO 7899-2.</p>
<p>1.6. Based on colony morphology, subculture individual colonies on blood agar or equivalent agar plates. Up to three colonies should be individually subcultured. If both presumptive <i>E. faecalis</i> (colony generally not surrounded by a halo) and <i>E. faecium</i> (colony generally surrounded by a halo) are observed, subculture at least one colony of each species separately.</p> <p>Incubate plates at 37 ± 1 °C for 18–24 h. Plates may be incubated for up to 48 h if required.</p>	<p>On Slanetz and Bartley agar presumptive <i>Enterococcus</i> colonies will appear raised with red, maroon or, sometimes, pink colour, either in the centre or throughout the colony.</p> <p>The number of subcultured colonies should be decided based on the laboratory's success rate at recognizing and isolating enterococci.</p> <p>It is recommended to subculture and store at least three colonies that exhibit a colony morphology typical for <i>Enterococcus</i>.</p>
<p>1.7. Select one of the subcultures and proceed with species identification (for details see section 2). If the first subculture is not identified as <i>E. faecalis</i> or <i>E. faecium</i>, proceed with testing the second and, if needed, the third subculture.</p>	<p>If presumptive colonies of both <i>E. faecalis</i> and <i>faecium</i> were identified and subcultured in step 1.6, perform species identification on isolates subcultured from both species.</p> <p>Initially, perform species identification on one subculture only. If this isolate is not confirmed as <i>E. faecalis</i> or <i>faecium</i>, proceed with testing the second and, if necessary, the third subculture. If none of the three subcultures is identified as <i>E. faecalis</i> or <i>faecium</i>, the sample may be considered negative.</p>
<p>1.8. Re-subculture one confirmed isolate (<i>E. faecalis</i> and/or <i>E. faecium</i>, as applicable) to avoid contamination. Incubate the plate(s) at 37 ± 1 °C for 18–24 h. Plates may be incubated for up to 48 h if required.</p> <p>Store the re-subcultured bacterial isolate(s) under appropriate conditions.</p>	<p>Isolates can be stored by suspending a loopful of colony material in a broth containing a cryoprotectant such as glycerol and storing the suspension at -80 °C. Alternative methods of storage may be used, provided that they ensure both the viability and the preservation of the isolate's properties.</p>
<p>2. Species identification (ID) of <i>E. faecalis</i> and <i>E. faecium</i></p>	

Procedure	Theory/comments
<p>2.1. It is always necessary to perform species ID on the presumptive <i>Enterococcus</i> isolates, as indicated above. The species ID should be conducted using an appropriate method.</p>	<p>Different laboratories may have different methods (biochemical tests, mass spectrometry, chromogenic agar, genotypic methods, WGS etc.) for performing species identification. Chromogenic/selective agar, such as bile-aesculin-azide (see e.g. ISO 7899-2), can be useful for distinguishing presumptive <i>E. faecalis</i> and <i>E. faecium</i> from other bacterial species that may have similar colony appearance on Slanetz and Bartley agar.</p> <p>For a detailed protocol on how to perform species ID by MALDI-TOF, please refer to the protocol “Identification of bacteria with MALDI-TOF (Matrix-Assisted Laser Desorption-Ionization Time Of Flight)”, which will be available on the EURL’s website.</p> <p>For a detailed protocol on how to identify <i>E. faecalis</i> and <i>E. faecium</i> by PCR, please refer to the protocol “PCR-based identification of <i>Enterococcus faecalis</i> and <i>Enterococcus faecium</i>”, which will be available on the EURL’s website.</p>
<p>3. Antibiotic susceptibility testing (AST)</p>	
<p>3.1. Perform antimicrobial susceptibility testing (AST), as detailed in the protocol “Susceptibility testing by MIC of bacteria from aquaculture animals”.</p>	<p>The protocol “Susceptibility testing by MIC of bacteria from aquaculture animals” will be available on the EURL-AMR’s website.</p>

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.
https://eur-lex.europa.eu/eli/dec_impl/2020/1729/oj/eng
- (2) Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food, EFSA Journal Volume 17, Issue 6, e05709, Jun 2019.
DOI: <https://doi.org/10.2903/j.efsa.2019.5709>
- (3) Technical specifications for a EU-wide baseline survey of antimicrobial resistance in bacteria from aquaculture animals. EFSA Journal Volume 22, Issue 7, e8928, Jul 2024.
DOI: <https://doi.org/10.2903/j.efsa.2024.8928>
- (4) Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation)
<https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02017R0625-20250105>
- (5) Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.
<https://eur-lex.europa.eu/eli/reg/2004/853/oj/eng>

APPENDIX 1

Composition and preparation of culture media and reagents

The buffered peptone water (BPW), Slanetz and Bartley agar and reagents are available from several companies. The composition of the dehydrated media given below is an example and may vary slightly among the different manufacturers. Note that the media should be prepared according to the manufacturer's instructions, if they differ from the description given here.

Buffered peptone water (Example)

<i>Formula</i>	<i>g/L</i>
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	9.0
Potassium dihydrogen phosphate (KH_2PO_4)	1.5
pH 7.0 +/- 0.2 @ 25°C	

Dissolve the components in water by heating if necessary. Adjust the pH so that after sterilization it is 7.0 +/- 0.2 at 25 °C. Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test. Sterilize by autoclaving at 121 °C for 15 minutes.

Slanetz and Bartley agar

Please refer to ISO 7899-2:200.

APPENDIX 2

FLOWCHART

for isolation of *Enterococcus faecalis* and *E. faecium* from mussels

Non-selective pre-enrichment [items 1.3-1.4]

25 g of sample in 225 mL of buffered peptone water
(incubate at 37 ± 1 °C for 18-22 h)



Isolation of presumptive *Enterococcus* [item 1.5]

Streak 10 µL of incubated pre-enrichment culture in BPW onto a **Slanetz and Bartley agar plate**.
(incubate at 37 ± 1 °C for 48 h)



Subculture of presumptive *Enterococcus* [item 1.6]

Subculture up to three individual presumptive colonies onto **blood agar plates** or similar.
(incubate at 37 ± 1 °C for 18-24 h and up to 48 h)



Identification and storage of isolates [section 2; steps 1.7-1.8]

Species ID by appropriate method
Subculture to ensure purity
Storage: Suitable method for keeping isolates viable [see item 1.8 for details].



Antibiotic susceptibility testing [section 3]