1.1- Water Sample collection

50mL (Fresh water) will be taken in a sterile falcon and transported to the lab as soon as possible (within 8 hours at most). For DNA extraction, at least 20 liters of water should be sampled. We recommend transportation at 4°C or on dry ice.

1.2- Inoculation

100µL of the water sample will be spread on following media:

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

The remaining sample will be kept at 4°C until the following day. If the CFU/mL cannot be determined from the first set of plates, ten-fold serial dilutions of the initial sample will be made using sterile water, up to 10^{-6}, and spread on the relevant plates (i.e. those where CFU/mL could not be determined using the first-day method).

1.3- Identification:

Gram staining will be performed on resistant isolates (at least four of each morphology). Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

1.4- Preservation of sample

The water samples will be preserved in 16-20% glycerol at -80C. Relevant colonies' morphology will be recorded and it is recommended that four colonies of each morphology from each treatment and sample are preserved in 16-20% glycerol at -80C, if needed later.

1.5- DNA extraction

Prior to DNA extraction, the water samples should be filtered in appropriately sized batches to result in accumulation of biomass on a 0.22 µm membrane. We recommend the use of sterile Sterivex filters with 0.22µm pore size with a polyethersulfone membrane (EMD MilliporeTM 122 SVGP01050). Shred the filters and extract the DNA from them using the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation); one filter per extraction tube. At least four tubes should be extracted according to the instructions.

Mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.

2.1- Soil sample collection

50 grams of top soil (7.5cm depth) will be collected in either zip lock sterile plastic bags or sterile falcon tubes. The samples will be transported to the lab as soon as possible (within 8 hours at most). We recommend transportation at 4°C or on dry ice.

2.2- Inoculation

Soil samples should be homogenized through vortexing for 30 sec in the sampled tube. If needed, the soil sample can be sieved to remove debris, roots etc. A subsample of the soil (several grams) should be set aside and frozen at -80°C analysis of soil properties. The dry weight and humidity content of this soil subsample can be determined by carefully weighting it before and after heading to 100°C for 24 hours. If desired, the sample can also be heated to 550°C to measure the volatile solids, but this is not required by this protocol.

1g of soil sample will be mixed with 9 mL of saline or PBS. The sample will be thoroughly shaken/vortexed before inoculation to resuspend the soil. The serial dilutions will be made and further inoculation will be done;

- Before the soil settles, remove 1 mL of the suspension with a sterile pipette and transfer it to 9 mL saline or PBS. Vortex thoroughly, and label as "B".
- Repeat this dilution step three times, each time with 1 mL of the previous suspension and 9 mL saline or PBS. Label these sequentially as tubes C, D, and E. This results in serial dilutions of 10^{-1} through 10^{-5} grams of soil per mL

100µL of each will be spread on the following plates **with the addition of *either* cycloheximide (50µg/ml) *or* nystatin (50 µg/ml) to avoid fungal growth**:

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

2.3-Identification:

Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

2.4- Preservation

- 6g of 15% glycerol/DESS to 4g of soil sample (wet weight, weight to weight) and store at -80C (Yu Chaewei, 2015).
- We recommend that all selected colonies are preserved in 16-20% glycerol at -80C.

2.5- DNA extraction

DNA will be extracted using the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation); using 0.25 g soil in each of two extraction tubes, according to the instructions.

Mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics

2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.

3.1- Sewage water collection

Influent water

Samples for bacteriological analyses will be collected directly into the plastic sample container. The sample container will be kept unopened until it is to be filled. The cap will be removed; a 1000 mL bottle will be held near the base and filled to within about one inch of the top without rinsing and recapped immediately. During sample collection, the sample container will be plunged with the neck partially below the surface and slightly upward. The mouth will be directed against the current flow. The sample will be transported to the lab as soon as possible (within 8 hours at most). We recommend transportation at 4°C or on dry ice.

Sewage water (manhole)

The sample container (1000 mL) will be lowered in the manhole and will be filled to within about one inch of the top without rinsing and recapped immediately. Minimum of duplicate samples will be collected from each site. Samples will be transported to the lab as soon as possible (within 8 hours at most). We recommend transportation at 4°C or on dry ice.

3.2- Inoculation

100µL of the sewage samples will be diluted in 100 mL saline or PBS. 100 µL of the resulting suspension will be spread on the following media:

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

The original sewage sample will be kept at 4°C until the following day. If the CFU/mL cannot be determined from the first set of plates, ten-fold serial dilutions of the initial sample will be

made using sterile water, up to 10^{-6}, and spread on the relevant plates (i.e. those where CFU/mL could not be determined using the first-day method).

3.3-Identification:

Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

3.4- Preservation

The 250mL bottle containing the sewage sample will be centrifuged at 10,000xg for 10min. The resulting pellet will be stored at −20 °C or −80 °C before DNA extraction and metagenomics analysis. It is recommended that four colonies of each morphology from each treatment and sample are preserved in 16-20% glycerol at -80C, if needed later.

3.5- DNA extraction

Prior to DNA extraction, the water samples should be filtered in 200 ml batches to result in accumulation of biomass on a 0.22 µm membrane. We recommend the use of sterile Sterivex filters with 0.22µm pore size with a polyethersulfone membrane (EMD MilliporeTM 122 SVGP01050). Shred the filters and extract the DNA from them using the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation); one filter per extraction tube. At least three tubes should be extracted according to the instructions.

Mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.

4.1- Treated wastewater (WWTP)

Treated wastewater samples will be collected in sterile 1L plastic bottles. Three bottles will be sampled from each site. These samples will be collected from each site, at the WWTP exit points (treated effluent) for each wastewater treatment plant (for influents, see the sewage protocol (point 3) above. The duration of treatment will be taken into account for the effluent samples in relation to the influent samples.
Samples will be transported to the lab as soon as possible (within 8 hours at most), or alternatively stored at 4°C at the WWTP until pickup. We recommend transportation at 4°C or on dry ice.

4.2 Inoculation

100µL of the sewage samples will be diluted in 9.9 mL saline or PBS. 100 µL of the resulting suspension will be spread on the following media:

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

The original effluent sample will be kept at 4°C until the following day. If the CFU/mL cannot be determined from the first set of plates, ten-fold serial dilutions of the initial sample will be made using sterile water, up to 10^{-6}, and spread on the relevant plates (i.e. those where CFU/mL could not be determined using the first-day method).

4.3- Identification:

Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

4.4 Preservation

The bottle containing the effluent or sewage sample will be centrifuged at 10,000xg for 10min, potentially in batches. The pellet will be stored at −20 °C or −80 °C before DNA extraction and metagenomics analysis. It is recommended that four colonies of each morphology from each treatment and sample are preserved in 16-20% glycerol at -80C, if needed later.

4.5- DNA extraction

Prior to DNA extraction, the water samples should be filtered in 500 ml batches to result in accumulation of biomass on a 0.22 µm membrane. We recommend the use of sterile Sterivex filters with 0.22µm pore size with a polyethersulfone membrane (EMD MilliporeTM 122 SVGP01050). Shred the filters and extract the DNA from them using the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation); one filter per extraction tube. At least four tubes should be extracted according to the instructions.

Mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.

5.1- Meat Sample collection

The meat sample (preferably, caecal content, carcass rinsate, carcass and cloacal swab, whole cut from retail meat) will be collected. The relevant information as described in GLASS protocol will be collected for each meat sample.

5.2- Inoculation

- for caecal content samples, mix 1 g ± 0.1 g of sample in 9 ml of cold 0.9% saline. Vortex for at least 20 s to ensure adequate suspension.
- for meat samples, mix 25 g ± 0.5 g of sample in 225 ml of cold 0.9% saline in a plastic bag that can be tightly closed (e.g. a Stomacher bag), A. Shake vigorously for 50 times.
- By using a pipette, transfer 100 µl from the suspension A into an Eppendorf tube containing 900 µl of cold 0.9% saline, B. Vortex well.
- Transfer 100 µl from the suspension B into an Eppendorf tube containing 900 µl of 0.9% saline. Vortex well.
- Proceed with serial dilutions until the desired final dilution (e.g. 10^{-6}).
- Once all the desired dilutions are ready, proceed with plating as follows.

Plate 100 µl of each relevant dilution should be plated to five media as follows;

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

5.3- Identification:

Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

5.4- Preservation

Suspension A will be stored at -80C by adding 16-20% glycerol. It is recommended that four colonies of each morphology from each treatment and sample are preserved in 16-20% glycerol at -80C, if needed later.

5.5- DNA extraction

DNA will be extracted using the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation); using 0.25 g caecal material in each of two extraction tubes, according to the instructions.

For meat samples, mix 25 g ± 0.5 g of sample in 225 ml of cold 0.9% saline in a plastic bag that can be tightly closed (e.g. a Stomacher bag), A. Shake vigorously for 50 times.

Remove 2x 50 mL of the liquid from the bag and put it into two falcon tubes. Centrifuge the tubes at 6000 rpm for 3 minutes. Remove the supernatant from each tube, and resuspend each pellet in 200 µL PBS. Move the pellets into separate DNA extraction tubes, and follow the kit instructions as for caecal material.

In both cases, mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.

6.1: Hospital Environment Swabs

Swab will be used for Hospital environment sampling. Diverse sampling will be done, including drains, wash basins, hospital surfaces, patient bed, floor, sitting benches etc. The swabs will be transported to the lab as soon as possible (within 8 hours at most). We recommend transportation at 4°C or on dry ice. Use several swabs for each setting, as many might be needed for the DNA extractions.

6.2- Inoculation

The inoculation will be performed by adding 1mL of PBS to the swab, vortex and streak the suspension on;

1. Mac Conkey agar (for the selection of Gram -ve)
2. Mannitol Salt agar (for the selection of Gram +ve)
3. Mac Conkey agar supplemented with 1mg/L of Cefotaxime for ESBL-producer screening
4. Mac Conkey agar supplemented with 0.25mg/L of Meropenem for presumptive carbapenemase-producers
5. Mac Conkey agar supplemented with 32mg/L of Temocillin for presumptive carbapenemase-producers
6. Mac Conkey agar supplemented with 0.125mg/L of Ciprofloxacin for screening of fluoroquinolone resistance.
7. BEA media for selection of enterococci

Plates will be incubated for 24hrs at 37±2°C, as well as 30±2°C and 20±2°C, if equipment for low-temperature incubation is available. CFU/mL will be determined.

6.3- Identification:

Based on Gram stain and colony morphology the bacteria will be further identified using biochemical tests. Based on biochemical tests, these will be categorized as oxidase-negative GNB, enterococci. These will be stored for later identification and further characterization.

6.4- Preservation

The charcoal swab will be stored at -80C. It is recommended that four colonies of each morphology from each treatment and sample are preserved in 16-20% glycerol at -80C, if needed later.

6.5- DNA extraction

Place the swab in 250 µL PBS and vortex it. Transfer the solution into a DNA extraction tube and follow the protocol for the Qiagen DNeasy PowerSoil Pro Kit (https://www.qiagen.com/nl/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/#orderinginformation). Several swabs are probably needed to obtain sufficient DNA.

Mix the resulting tubes, and measure the DNA concentrations.

If the total amount of DNA > 15 µg, divide samples according to the following priority order:

1) One tube with 2 µg DNA for shotgun metagenomics
2) One tube with 2 µg DNA for qPCR
3) One tube with 10 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 7 µg and 15 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One tube with 5 µg DNA for functional metagenomics
4) One spare tube with the remaining material

If the total amount of DNA is between 2 and 7 µg, divide samples according to the following priority order:

1) One tube with 1 µg DNA for shotgun metagenomics
2) One tube with 1 µg DNA for qPCR
3) One spare tube with the remaining material

If the total amount of DNA < 2, divide the samples evenly into two tubes: one for DNA for shotgun metagenomics and for qPCR.

Note the DNA weight and concentrations for every tube.