TENTATIVE STANDARD OPERATING PROCEDURE for:

Department of Applied Technology, Institute of Applied Sciences and Technology, National Centre of Research and Technology, Burkina Faso; **Food Research Institute**, Council for Scientific and Industrial Research, Ghana; **University of Development Studies**, **Department of Applied Biology**, Ghana; **University of Abomey-Calavi**, **Faculty of Agricultural Science**, Benin, under the GreenGrowth Project

Sampling and identification of microbial isolates from fermented food products

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1. Scope and objective

This procedure is in its original form made as a part of the project Preserving African Food Microorganisms for Green Growth (DFC No. 13-04KU).

This procedure has two purposes:

i. to describe sampling and isolation of microorganisms (*Bacillus* spp., lactic acid bacteria (LAB), yeasts, a.o.) from fermented food products and raw materials/ingredients used for the processing of the fermented foods
ii. to describe identification of the isolated microorganisms

This procedure applies to all new research/food quality control activities carried out as part of the GreenGrowth project at:

- Department of Applied Technology, Institute of Applied Sciences and Technology, National Centre of Research and Technology, Burkina Faso
- Food Research Institute, Council for Scientific and Industrial Research, Ghana
- University of Development Studies, Department of Applied Biology, Ghana
- University of Abomey-Calavi, Faculty of Agricultural Science, Benin

This procedure is connected to the other standard operating procedure developed under the GreenGrowth project on: "Handling, maintenance and preservation of microbial isolates in culture-collection" [SOP-GG-01-00], which is being referred to whenever applicable.

Financial support provided by Danida, Ministry of Foreign Affairs, Denmark, through the GreenGrowth project is gratefully acknowledged.

This procedure has the document ID: SOP-GG-02-00. The procedure should be reviewed for updating every two years.

2. Sampling of isolates

Sampling is an operation that requires the most careful attention; emphasis cannot be too strongly laid on the necessity of obtaining a properly representative sample of the examined products (ISO/707:2008).

Heterogeneity in foods provides particular challenges for sampling. Sampling uncertainty is mainly influenced by the heterogeneity of the sample, the sample size and the sampling method.

The procedures for sampling described here are good practices to be followed whenever practicable. It is, however, impossible to put forward fixed rules on sampling to be followed in every case, and, unforeseen circumstances may render some modifications in sampling procedures desirable. Whenever special requirements are given for sampling and/or arise from a specific analysis to be performed, these should be followed (ISO/707:2008).

The section on sampling described in this procedure is based on the two ISO standards, ISO/TS/17728:2015 and ISO/707:2008.

2.1 Sampling plan

- A sampling plan should be established, describing a predetermined procedure for the selection, withdrawal, and preparation of samples from a fermented food product/raw materials/ingredients used in the fermentation process/samples from final fermented food products to ensure a correct sampling yielding the required information
- The sampling plan is recorded in the sampling report [F/2.01/2017]. The sampling report ID as well as sample IDs are recorded in [SOP-GG-01-00; electronic accession file [F/3.03/2017] and print out accession form [F/3.04/2017]]

2.2 Sampling report (sampling report ID)

Samples should be accompanied by a sampling report, which should include the information described below, adopted from ISO/707:2008. The sampling report allows traceability of the samples.

- Sample report ID
- Entry ID [SOP-GG-01-00, section 3.2]
- Name of product [SOP-GG-01-00, section 3.2]
- Name of production site/shop, including address and contact information [SOP-GG-01-00, section 3.2]
- Sample type; raw material/ingredient, fermentation sample, final product (solid, semi-solid, liquid) [section 2.4]
- Date for sampling [section 2.2]
- Name of sampling personnel [section 2.2]
- Flow diagram for processing [section 2.4]
- Storage/transport of samples to the laboratory [section 2.4]

- Sampling time points (raw material/ingredient, fermentation sample and hour of withdrawal, final products etc.) [section 2.4]
- Total number of samples [section 3.3]
- Sample IDs [section 2.3]
- Sample mass/volume [section 3.3]
- Sampling procedure
- Sampling details

See [F/2.01/2017] for an example of the sampling report set up.

The **sampling report ID** is assigned as follows:

- The initials of the institute; DTA (Départment Technologie Alimentaire, Burkina Faso), FRI (Food Research Institute, Ghana), UDS (University for Development Studies, Ghana), UAC (University of Abomey-Calavi, Benin), etc.
- Entry ID [SOP-GG-01-00, section 3.2]; 01,02,03,…10,11…100,101, etc.
- Sampling date (DD/MM/YYYY)

Example of **sampling report ID**: DTA-01-05/05/2017 (i.e. a sampling performed by DTA with entry ID 01 at the 5th of May 2017).

2.3 Sample ID

To facilitate traceability of each sample from the examined fermented food product (e.g. isolates from raw materials/ingredients used for the fermentation, isolates sampled during the fermentation of the food product and isolates from final fermented food products), **each sample is assigned a unique sample ID, which is permanently bound to the sample**.

The **sample ID** is assigned as follows:

- Entry ID [SOP-GG-01-00, section 3.2]; 01,02,03…10,11,…100,101, etc.
- Sample type abbreviation; R = raw material, I = ingredient, F = fermentation sample, P = final product, T = type strain (reference strain). The time (hour) of sample withdrawal is included whenever applicable
- Number of the sample within the sample type, continuous increasing numbers assigned for each sample type (R,F,P,T), in case multiple samples are withdrawn from a sample type e.g. several different raw materials or final products are sampled

Examples of **sample ID**:

Sample ID	Explanation
01-R-02	(i.e. a sample belonging to entry ID 1, withdrawn from raw material, second sample)

01-F6-01	(i.e. a sample belonging to entry ID 1, withdrawn at fermentation hour 6, first sample)
01-F12-01	(i.e. a sample belonging to entry ID 1, withdrawn at fermentation hour 12, first sample)
01-P-03	(i.e. a sample belonging to entry ID 1, withdrawn from final product, third sample)
02-T-01	(i.e. a sample belonging to entry ID 2, comprising a type strain, first sample)

2.4 Sampling and handling of samples

This section describes the different aspects to be considered when sampling of a fermented food product is planned, and should be consulted when preparing the sampling plan. The section is adopted from ISO/TS 17728:2015

Principles and general requirements

- Sampling techniques applied shall not modify the intrinsic microbiota of the product (such as via contamination from sampling equipment of the environment)
- Sample equipment and sample containers should be sterilized (121°C for 15-20 min) or decontaminated using 70% (v/v) alcohol followed by flaming. Pre-sterilized equipment could also be used
- Samples for microbiological examinations should always be taken first using aseptic techniques. Whenever possible they should be taken from the same product containers as for chemical and physical analyses

Sampling sites and description of the processing

- Samples should be obtained from two different production sites
- Sampling should be performed at least twice at each production site at two separate occasions
- Flow diagrams of the processing and picture documentation should to the extent possible be established

Materials to be sampled

- The raw materials and all ingredients added during processing
- The product during fermentation
- The final product
- Optional, the final product bought on markets/shops

Product types

- For solid products/raw materials/ingredients, samples should be taken stratified at different parts of the product surface and centre, which should subsequently be analysed separately. If natural occurrence or variation of the microbiota in a solid product is to be analysed, then three stratified surface samples and three stratified centre samples are analysed separately. It is important to include how the sample was withdrawn from the solid product, as a drawing in the sampling report
- For liquid products/raw materials/ingredients, initial homogenisation of the product should be performed before the required volume is withdrawn from the container

- For analyses of final products: If the product is packaged, the packaging should be cleaned with 70% (v/v) ethanol, to avoid contamination from the environment

Mass/volume of samples

- The minimum quantity required for all tests included in the examination is determined
- If the product/raw material/ingredient contain coarse particles, it might be necessary to increase the minimum sample size to ensure a representative sample of the product/raw material/ingredient

Sampling time points

- The sampling time points should be identified representing the different steps in the processing, from the raw material(s) to the final product
- During the fermentation step in the processing it is important to include the start point i.e. 0h and end point of the fermentation. If several fermentation steps are included in the processing, also start point and end point of subsequent fermentation steps should be sampled. Sampling during the fermentation should be performed continuously; for example at hour 0, 4, 8, 12, 16, 24, 36, 48, 72 after the start point of the fermentation

Handling of obtained samples

- Storage and dispatch of samples should be such that the state of the sample at the time of sampling remains essentially unaltered until the time of starting the test procedure
- The collected samples should be kept in an icebox with ice and taken to the laboratory where samples are transferred to the refrigerator at 5°C ± 2°C for analyses within a few hours
- The time for dispatch of the samples to the testing laboratory should be as short as possible

Chemical and physical analyses:

- For each sample measure the temperature, pH and titritable acidity (if relevant)
- For solid products, additionally the water activity and moisture content can be recorded

3. Enumeration of microorganisms from samples

In order to enumerate the microorganisms in the samples withdrawn from food fermented products, the microorganisms are cultivated on agar plates to give a viable count of the microorganisms present in the analysed sample. In many cases a high number of microorganisms are present, bringing about the need for dilution before enumeration can be performed. Dilution is described for liquid products/raw materials/ingredients [section 3.1] and solid products/raw materials/ingredients [section 3.2]

3.1 Dilution series for liquid products/raw materials/ingredients

- A sample is taken directly from the homogenized liquid product, representing undiluted sample (10^0), if low numbers of microorganisms occur in the product
- Serial 10 fold dilutions are prepared by transferring 1.0 mL of homogenized liquid sample to 9.0 mL of sterile saline peptone water (SPO) 10^{-1} dilution. SPO pH 5.6 is used for LAB and yeasts, whereas SPO pH 7.2 is used for *Bacillus* spp.
- Repeat until a suitable dilution is reached
- Prepare duplicate dilution series
- Use SPO 4% (w/v) NaCl for salty foods and SPO 10% (w/v) sucrose for high sugar food products
- Perform one/duplicate plating(s) for each dilution series prepared for enumerations as described in [section 3.3] (LAB), [section 3.4] (aerobic endospore-forming bacteria), [section 3.5] (yeasts)

3.2 Dilution series for solid and semi-solid products/raw materials/ingredients

- A 10.0 g sample of the food product is taken, representing either surface or interior
- The weighing is done directly into a Stomacher bag, to which sterile saline peptone water (SPO) is subsequently poured until the weight reaches 10 times the weight of the sample (total weight should be 100.0 g if the sample size is 10.0 g). SPO pH 5.6 is used for LAB and yeasts, whereas SPO pH 7.2 is used for *Bacillus* spp.
- Homogenize for 30 seconds at normal speed
- Homogenized sample + SPO constitute the 10^{-1} dilution
- Serial dilutions are prepared by transferring 1.0 mL of sample to 9.0 mL of SPO in a test tube to achieve a 10X dilution. SPO pH 5.6 is used for LAB and yeasts, whereas SPO pH 7.2 is used for *Bacillus* spp.
- Repeat until the suitable dilution is reached
- Prepare duplicate dilution series
- Use SPO 4% (w/v) NaCl for salty foods and SPO 10% (w/v) sucrose for high sugar food products
- Perform one/duplicate plating(s) for each dilution series prepared for enumerations as described in [section 3.3] (LAB), [section 3.4] (aerobic endospore-forming bacteria), [section 3.5] (yeasts)

NOTE: for solid raw material, the 10.0 g product can be soaked for maximum 30 minutes in 90.0 g SPO (pH 5.6 for LAB and yeasts, SPO pH 7.2 for *Bacillus* spp.) (placed on ice) before the raw material is homogenized (for 30

seconds at normal speed) or manually by hand. This solution is used for the preparation of serial dilutions, as described above.

3.3 Enumeration of lactic acid bacteria (LAB) counts

Adapted from ISO 15215:1998

LAB:

- Perform one/duplicate plating(s) for each dilution series prepared for enumerations of LAB
- Man, Rogosa and Sharpe (MRS), pH 6.2 agar should be used as medium for enumeration of LAB
- Pour plate technique should be used for plating; 1.0 mL from the appropriate dilutions is added to a petri dish followed by 15-20 mL MRS agar, pH 6.2, of 45°C ± 1 °C
- Petri dishes are incubated inverted under anaerobic conditions, i.e. in an anaerobic jar with anaerocult A
- Incubated at 30°C or 37°C for up to 3-5 days
- For CFU determinations plates, with 25 to 200 colonies are used
- Calculate the CFU/mL for liquid samples and CFU/g for solid samples

Lactobacillus spp.:

- Perform one/duplicate plating(s) for each dilution series prepared for enumerations *Lactobacillus* spp.
- MRS agar pH 5.4 should be used as a selective media for *Lactobacillus* spp.
- Pour plate technique should be used for plating; 1.0 mL from the appropriate dilutions is added to a petri dish followed by 15-20 mL MRS agar, pH 5.4, of 45°C ± 1 °C
- Petri dishes are incubated inverted under anaerobic conditions, i.e. in an anaerobic jar with anaerocult A
- Incubated at 30°C for 3 days
- For CFU determinations, plates with 25 to 200 colonies are used
- Calculate the CFU/mL for liquid samples and CFU/g for solid samples

Streptococci and lactococci:

- Perform one/duplicate plating(s) for each dilution series prepared for enumerations streptococci/lactococci
- M17 medium with 0.5% (w/v) lactose should be used for fastidious LAB (streptococci and lactococci)
- Spread plate technique should be used for plating; 0.1 mL from the appropriate dilutions is spread over the surface of MRS, pH 6.2, agar. Resulting in 10X dilution upon plating, which should be included when calculating the CFU
- Petri dishes are incubated inverted at aerobic conditions
- Incubated at 30°C or 37°C for 3 days
- For CFU determinations, plates with 25 to 200 colonies are used
- Calculate the CFU/mL for liquid samples and CFU/g for solid samples

NOTE: the time for pour plating, should not exceed 45 minutes after performing the dilutions (remember to whirley mix each dilution before pour plating). The time before mixing the sample with the agar should not exceed 20 min.

3.4 Enumeration of mesophilic aerobic counts/aerobic endospore-forming bacteria (AEB)

Adapted from ISO 4833:2003

- Perform one/duplicate plating(s) for each dilution series prepared for enumerations of aerobic endospore-forming bacteria (AEB) (e.g. *Bacillus* spp.)
- Plate count agar (PCA) or nutrient agar (NA) should be used as media for enumeration of (AEB)
- For analyses focusing on AEB, enumeration of spore/dead vegetative cells is performed by heat-treatment of 10^{-1} dilution sample, whereas spores/viable vegetative cells are enumerated from a non-heat-treated dilution series. Hence two dilution series are prepared
- Heat-treatment is performed by:
    - Transferring 3.0 mL of the 10^{-1} dilution to a 10 mL test tube
    - Incubating the test tube for 10 min at 80°C in a water bath (cool on ice after heat treatment)
- Serial dilutions of the heat-treated and non-heat treated samples are prepared by transferring 1.0 mL of sample to 9.0 mL of sterile saline peptone water (SPO) pH 7.2 contained in a test tube to achieve a 10X dilution
- Repeat until the suitable dilution is reached
- Surface plate technique should be used for plating; 0.1 mL from the appropriate dilutions is spread over the surface of PCA or N agar, of 45°C ± 1 °C. Resulting in 10X dilution upon plating, which should be take into account when calculating the CFU
- Petri dishes are incubated inverted at aerobic conditions
- Incubated aerobically at 30°C for 1 to 3 days
- For CFU determination, plates containing 25 to 200 colonies are used
- Calculate the CFU/mL for liquid samples and CFU/g for solid samples

3.5 Enumeration of yeast counts

Adapted from ISO 7954:1988

- Perform one/duplicate plating(s) for each dilution series prepared for enumerations of yeasts
- Malt yeast glucose peptone agar (MYGP), pH 5.6 should be used as media for enumeration of yeasts
- For products where a high number of e.g. LAB is expected, chlorotetracycline (50 mg/L) and chloramphenicol (100 mg/L) can be added to the MYGP agar
- Spread plate technique should be used for plating; 0.1 mL from the appropriate dilutions is spread over the surface of MYGP, pH 5.6, agar. Resulting in 10X dilution upon plating, which should be taken into account when calculating the CFU
- Petri dishes are incubated inverted at aerobic conditions
- Incubated at 25°C (or 30°C) for 3-5 days
- For CFU determinations, plates with 20 to 50 colonies are used

- The presence of mould should be noted
- Calculate the CFU/mL for liquid samples and CFU/g for solid samples

4. Selection and purification of isolates

Random selected isolates are selected for further analyses using the enumeration plate after CFU calculations has been performed [section 3]. How random selection and purification of isolates are performed is described in the following.

4.1 Random selection of isolates

For each sample obtained as part of the sampling plan, the highest dilution with suitable number colonies of bacteria/yeasts (25-200 bacterial colonies, 20-50 yeast colonies) will be used for the random isolation:

- Petri dishes with MRS or M17 for isolation of LAB and fastidious LAB (streptococci, lactococci)
- Petri dishes with PCA or NA for isolation of *Bacillus* spp.
- Petri dishes with MYGP for isolation of yeasts, containing chlorotetracycline (50 mg/L) and chloramphenicol (100 mg/L), if high numbers of LAB is expected in the sample

Optimally 20-25 colonies should be randomly picked from a given sample, see Fig 1. Preferably all isolates originate from one dilution plate (petri dish). If no plates contain 20-25 appropriate colonies, then two or more dilution plates are used. Randomly picked isolates are purified [section 4.2].

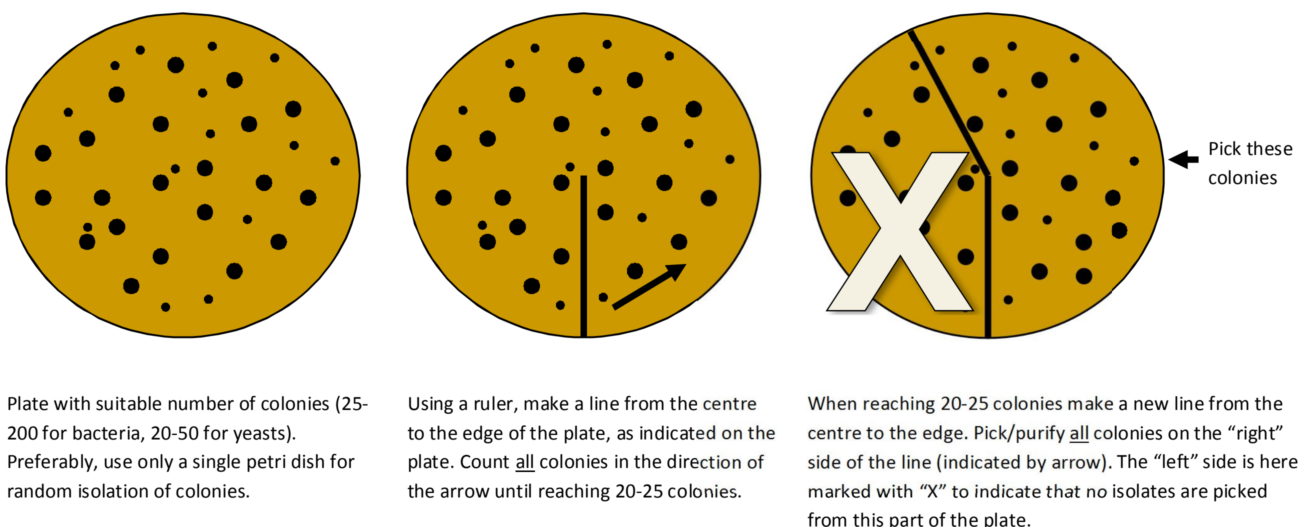

Fig 1: Schematic presentation of the procedure for random picking of isolates from a petri dishe.

4.2 Purification of selected isolates

In order to obtain pure cultures comprising a single microorganism, the randomly picked colonies should be purified by transferring colonies to grow in liquid substrate followed by successive streaking on solid media until pure cultures are obtained.

Purification is performed as described in the following:

- The randomly selected colonies from the dilution plates as described in [section 4.1] are used
- Transfer single colonies of each of the randomly selected colonies to separate test tubes with 10 mL of sterile appropriate broth in a sterile tube whirly mix and incubate at the appropriate time and temperature, Table 1
- After growth, streak the culture on appropriate solid substrate as shown in Fig. 2
- Incubate the plates for the appropriate time, temperature and condition, Table 1
- After growth, one pure colony is streaked again on solid substrate to confirm the purity of the isolate
- Pure isolates have uniform colony characteristics and are free on any contamination
- Remember to label purification plates and tubes with the isolate code [SOP-GG-01-00, section 3.3], dilution and the plate it was picked from

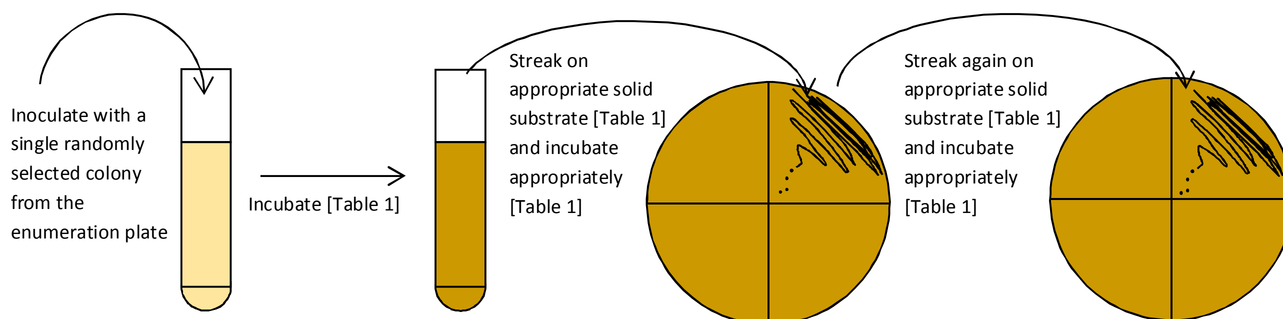

Fig. 2. Schematic presentation of procedure for purification test of an isolate. An isolate grown in the appropriate broth is streaked onto ¼ of the appropriate agar plate, to obtain single colonies on the agar plate upon incubation.

Table 1: Media and incubation conditions for the different groups of microorganisms. Media composition and preparation methods [A/03/2017].

Microorganism	Media	Condition on agar	Temperature	Incubation time (broth)	Incubation time (agar)
LAB	MRS, pH 6.2	Anaerobic	30°C	24h-48h	3-5 days
Lactobacillus spp.	MRS, pH 5.4/6.2	Anaerobic	30°C	24h-48h	3 days
Lactococci and streptococci	M17 + 0.5% (w/v) lactose	Aerobic	30°C or 37°C	24h-48h	3 days
Bacillus spp.	Plate count or Nutrient	Aerobic	30°C	24h-48h	1-3 days
Yeasts	MYGP	Aerobic	25°C or 30°C	24h-48h	3-5 days

5. Identification of isolates

All isolates deposited in the GreenGrowth culture-collection should be identified as part of the documentation. This section comprises descriptions of the different steps in the identification process. All data obtained during the identification of the isolates are included in [SOP-GG-01-00; accession file [F/3.03/2017] and the accession form [F/3.04/2017]].

5.1 Phenotypic identification

The different tests, which should be performed as initial phenotypic characterization of pure isolates is listed in Table 2. The obtained results are recorded in [SOP-GG-01-00; accession file [F/3.03/2017] and the accession form [F/3.04/2017]].

Table 2: Phenotypical tests of microorganisms

	Microorganism		
Phenotypical tests	**LAB**	***Bacillus* spp.**	**Yeasts**
5.1.1. Macro-morphology (colony morphology on solid substrate)	x	x	x
Colour, size, shape, surface, margin, profile [A/04/2017]			
5.1.2.Macro-morphology in liquid substrate	x	x	x
Sediment, pellicle/surface layer [A/04/2017]			
5.1.3 Micromorphology/Cell morphology			
Shape, cell arrangement	x	x	x
Vegetative reproduction, mycelium			x
Spore morphology [A/04/2017][a]		x	x
Spore position		x	x
5.1.4 Other characteristics [I/5.01/2017]			
Gram test	x	x	
Catalase test	x	x	
Oxidase test	x	x	
CO_2 production	x		
5.1.5 Biochemical tests			
Fermentation of carbohydrates [I/5.02/2017]	x	x	x
Assimilation of carbohydrates [I/5.03/2017]			x
Assimilation of nitrate [I/5.04/2017]			x

[a] To enhance sporulation, presumptive *Bacillus* spp. can be grown in nutrient broth added $MnCl_2$ (50 mg/L) before microscopic examination. To enhance ascospore formation of yeasts they can be grown on acetat agar at 25°C for 7-10 days before microscopic examination.

Other basic tests can be also applied i.e. growth in 10% NaCl (w/v), growth at 50°C, anaerobic growth etc.

5.2 Initial grouping of isolates

In cases where many isolates has been ontained as part of the examination of a fermented food product, initial grouping of isolates is carried out to allow identification of all isolates. Initial grouping of isolates facilitated through Rep-PCR/GTG_5 fingerprinting followed by clustering using Bionumerics as described in [I/5.05/2017].

DNA used for the Rep-PCR/GTG_5 fingerprinting is extracted using either of the following methods:

- Instagene kit (BioRad), following the instructions of the manufacturer for bacterial DNA extraction (which also work for yeasts). **NOTE:** use only 100 µL Instagene mixture instead of 200 µL as stated by the manufacturer. Extracted DNA can be stored at -18°C for a least one year
- For bacteria only: streak the isolate on the surface appropriate agar and incubate at room temperature (25°C) for 18 h, at appropriate conditions [Table 1, p. 10]. After incubation, one loopfull of bacterial colony mass is suspended in 250 µL of 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), incubated at 100°C for 10 min and centrifuged at 15,000 g for 10 min. The supernatant containing the DNA is collected in a fresh Eppendorf tube and kept at 4°C/-18°C until use

NOTE: Rep-PCR is only suitable for grouping isolates that have been partially characterized (e.g. catalase, Gram-reaction and microscopy [I/5.04/2017] and Table 2, p. 11). A suitable group for Rep-PCR would for instance be Gram-positive, catalase-negative rods and cocci originating from MRS (i.e. presumptive LAB).

5.3 Molecular identification

Molecular identification of LAB and yeasts is performed by sequencing of 16S rRNA gene for bacteria and D1-D2 region of 26S rRNA gene for yeasts. Amplification of 16S rRNA gene and D1-D2 region of 26S rRNA gene is described in [I/5.06/2017].

NOTE: molecular identification of *Bacillus* spp. comprises other steps than for LAB and yeasts. Description of *Bacillus* identification can be found in “Protocol for the identification of Bacillus species. SeedFood/KU-LIFE/LITH 2010”.

6. Enclosures

Forms
[F/2.01/2017] Sampling report

Instructions
[I/5.01/2017] Gram test, catalase test, oxidase test and test for CO_2 production
[I/5.02/2017] Fermentation of carbohydrates
[I/5.03/2017] Assimilation of carbohydrates by yeasts
[I/5.04/2017] Assimilation of nitrate by yeasts
[I/5.05/2017] Initial grouping of microorganisms for identification –Rep-PCR
[I/5.06/2017] Molecular typing of LAB and yeasts by amplification and sequencing of 16S RRNA gene or 26S rRNA gene (D1/D2 region)

Attachments
[A/01/2017] Solution and media
[A/02/2017] Colony morphology
[A/03/2017] Wickerham card (for print out

NB: enclosures are not included