African Food Tradition rEvisited by Research
FP7 n°245025

Start date of project: **01/09/2010**
Duration: **45 months**

Deliverable number: D1.2.3.7

Title of deliverable: SOPs for the isolation and selection of potential starter cultures for groups 1 and 2

Deliverable type (Report, Prototype, Demonstration, Other): Report

Dissemination level (PU, PP, RE, CO)*: PU

Contractual date of delivery: February 2011

Actual date of delivery: September 2011

Work-package contributing to the deliverable: WP 1

Organisation name of lead contractor for this deliverable: UAC

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This document has been send to :

The coordinator by WP Leader	Date: September 2011
To the Commission by the Coordinator	Date: October 2011

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SOPs for the isolation and selection of potential starter cultures for groups 1 and 2

SOP Number: **MICRO**-11

Date of creation: 03/05/2011	Date of revision: 26/09/2011

Deliverable type (Report, Prototype, Demonstration, Other): Report

Dissemination level (PU, PP, RE, CO)*: PU

Contractual date of delivery: month 6 / Actual date of delivery: month 13

Work-Package contributing to the Deliverable: WP1

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Key-words: akpan, gowé, kenkey, kiskh, lanhouin, kitoza, potential starter culture, lactic acid bacteria, yeast, coagulase negative staphylococci

Table of contents

1 SCOPE AND APPLICATION

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong).

2 POTENTIAL STARTERS STRAINS

Lactic acid bacteria (LAB) will be considered as potential starter culture for Group 1 Cereal based products and Group 2 Meat and Fish products.

Coagulase negative staphylococci (SCN) will be considered as potential starter culture for Group 2: Meat and Fish products

Yeasts will be considered as potential starter culture for Group 1 Cereal based products.

3 SELECTION

LAB will be isolated from selective MRS agar at pH 5.7 (as described in the norm AFNOR NF V 04-503, SOP Micro-10), or M17 or Rogosa agar if relevant. The media could be inoculated either by 0.1 ml of the dilution spread on the selective agar media surface and incubated in an anaerobic chamber or by 1.0 ml of the dilution recovered by 15 ml of the medium agar. The incubation is at 30°C for 72h.

CNS will be isolated either from selective Baird-Parker agar (as described in NF EN ISO 6888 for coagulase positive staphylococci, SOP-Micro-05) or from selective Mannitol Salt agar incubated in aerobic condition (0.1 ml spread on the surface agar media) at 30°C for 18 to 48h.

Yeasts will be isolated from media described in International Standard – NF ISO 7954 (SOP Micro-09).

20% of the colonies on the respective selective plates that contain less than 150 colonies will be picked up randomly. Morphological observation, catalase and gram staining will be performed. The pure strains will be stored frozen until their identification.

4 IDENTIFICATION

Accurate identification of the isolates of LAB, CNS and Yeasts at the species level will be carried out by molecular approaches for those that will be developed as starters.

When relevant, characterisation at the strain level (specific profile) will be done by PFGE, RAPD or others methods.

5 PROPERTIES OF LAB (FOR GROUPS 1, 2)

5.1 Technological properties

Acidification potential

Biomass

Resistance to drying

Flavour potential for products having specific flavours (Kishk)

Antibacterial activity

Production of exopolysaccharides (slime, only for Group 1)

Amylolytic property (only for Group 1: Akpan, Gowé, Kenkey)

Peptidasic activity (only for Kishk product)

5.2 Safety criteria

Absence of transferable antibiotic resistance

Absence of amine production

6 PROPERTIES OF CNS (FOR GROUP 2)

6.1 Technological properties

Resistance to acidity

Biomass

Resistance to drying

6.2 Safety criteria

Absence of enterotoxin genes

Absence of transferable antibiotic resistance

Absence of DNase activity

Absence of Hemolysin activity

7 PROPERTIES OF YEAST (FOR GROUP 1)

Promote the growth of LAB

Flavour profile

8 REVISION RECORD

Date	Responsible person	Description of change
13/09/2011	**Meeting group**	

9 APPENDIX FOR TECHNOLOGICAL PROPERTIES OF LAB AND CNS

9.1. Determination of acidification activity (LAB)

The acidification rate will be calculated as ΔpH. The pH of tested strains will be determined in RSM using a pH meter. A portion of 50 ml RSM in 100 ml Scott bottle held at 32° and 37° C, for mesophilic and thermophilic cultures respectively, will be inoculated with 2% of culture at early stationary growth phase. The pH will be measured at 0, 2, 4 and 6h. The ΔpH will be calculated as ($\Delta pH = pH_{atT\ time} - pH_{at\ zero\ time}$). The cultures will be considered fast, medium and slow acidifying when a Δ pH of 0.4 unit will be achieved after 3, 3-5 and > 5h respectively.

9.2. Efficiency of biomass separation (LAB, CNS)

The absorbance at 650 nm of the supernatants that resulted after the centrifugation of the growth media will be used to express the biomass separation. Absorbance ($A_{650\ nm}$) with a range of 0 – 0.1 unit `indicates a good separation of biomass while absorbance of 0.2 to 0.3 and more than 0.3 unit indicate fair and poor biomass separation, respectively.

For CNS, only the biomass production will be followed in different culture media by absorbance ($A_{650\ nm}$).

9.3. ***Stability of lyophilized cultures (LAB, CNS)***

The obtained pellets in the biomass separation experiments will be resuspended in RSM fortified with 7 % w/v saccharose. Five cryoscopic vials containing 5 ml, of inoculated milk, each will be subjected to freeze-drying by use of a Freeze-dry system. The temperature program will be set as follow: 4h at –32°C; 10h at –15°C; 5h at 10 °C; 5h at 25°C. After lyophilization (zero time) one vial will be taken to determine the acidification activity of the culture. Two of the remaining four vials will be stored at -80°C for further use. The remaining two vials will be kept at 10°C and analyzed by the acidification test after 15 and 30 days, respectively. Untreated cultures (non-freeze-dried) will be used as control. The acidification activity of treated (freeze-dried) and untreated cultures will be followed in RSM at different time intervals 0, 2, 4 and 6h as described below. According to the effect of lyophilization on

the culture stability, the cultures will be grouped into three classes (good, ≤ 1h; fair, ≤ 1.5h; poor; > 2h) depends on the difference in the required time needed to drop the pH of reconstituted milk by 0.4 unit of the freeze-dried and unfreeze-dried culture.

9.4. Evaluation of flavor development (LAB) (for Kishk)

The ability of tested strains to develop flavor will be evaluated in milk. Fermented milk will be prepared by adding 3% of active single tested strain into sterilized milk. After 6h of incubation at optimum growth temperature for the tested strain, the fermented milk will be kept at 4°C for 18h. Final products will be evaluated for flavor and texture by 9 trained panellists.

9.5. Screening for antagonistic activities (LAB)

Antagonistic activity will be examined as described by Geis et. al. (1983). Cultures will be propagated in M17 or MRS medium and incubated at optimum temperatures for 16h. The 16-h old culture will be spotted (5µl) onto appropriate agar medium. The plates will be incubated at optimum temperature for 48h then overlaid with soft agar medium (0.8% agar) inoculated with indicator organism at a concentration of $\log_{10}$ 7 CFU/ml. After 24 h, plates will be examined for zones of inhibition surrounding individual colonies. Cultures belong to the same genus will be interacted together. Each tested strain will be applied as inhibitor organism while other one will be taken as indicator organisms.

9.6. Determination of slimy production (LAB, group 1)

Cultures will be plated on the M17 or MRS medium and incubated at optimum temperatures for 24h. The slime formation will be determined by use of inoculated loop method. Formed colonies will be dragged up using a metal loop and strains will be considered positively slimy producer if the length of the slime will be above 1.5 mm.

9.7. Determination of peptidase activity (LAB from Kishk)

The strains will be cultivated and centrifuged. The obtained pellets will be washed twice in potassium phosphate buffer (10 mmol/l, pH 7.0), resuspended in the same buffer and diluted to A_{650}.= 1.0 (*ca*. 100 mg cell wet weight/1 ml). Cell permeabilization will be carried out as described by Miozzari et al (1978). A portion of 100 µl of toluene will be added to 1 ml the culture suspension, mixed and frozen at –20°C for 15h. Aminopeptidase activity of the attenuated cells will be measured using leucine para-nitroanilide (Sigma, St. Louis, MO) as a substrate according to the method of El-Soda and Desmazeaud (1982). One unit of aminopeptidase activity will be defined as the amount of enzyme producing a change of 0.01 unit/min of absorbance at A_{410} and 37 °C. The specific activity will be defined as the number of activity units/one OD at 650 nm of the bacterial cells in the cultivated culture. (El-Soda and Desmazeaud 1982).

10 APPENDIX FOR SAFETY CRITERIA FOR LAB AND CNS

10.1. Determination of antibioresistance (LAB, CNS)

This determination will be focussed on Tetracycline, Erythromycin and Penicillin as antibioresistance for both bacterial group has been described associated to mobile genetic element (plasmid, transposon) (review Talon and Leroy 2011).

Antibiotic susceptibilities will be evaluated by the disk diffusion method with Mueller Hinton agar for CNS and MRS or M17 for LAB according to the guidelines of the CA-SFM (CA-SFM, 2007) or National Committee for Clinical Laboratory Standards (NCCLS 2002). The following antimicrobial susceptibility test disks will be used: erythromycin (15 μg), penicillin G (6 μg), and tetracycline (30 μg). Inhibition zones will be measured precisely to a millimeter and the resistance or susceptibility of the antibiotics will be interpreted as suggested by the CA-SFM standards (2007) or NCCLS (2002).

10.2. Determination of amines production (LAB)

For biogenic amine production, strains will be cultivated at 30°C for 24h to 48h in media supplemented with 0.05g/L pyridoxal-5-phosphate and 2.5g/L of each of the following precursor amino acids: L-histidine monohydrochloride, L-ornithine monohydrochloride, L-lysine monochloride, L-phenylalanine, L-tryptophan, L-tyrosine disodium salt (Bover-Cid and Holzapfel, 1999). Biogenic amines will be analysed in filtered supernatants by reverse-phase liquid chromatography of their dansyl derivates prepared as described previously (Hernandez-Jover et al; 1996).

10.3. Determination of enterotoxin genes (CNS)

Nine major antigenic types of staphylococcal enterotoxins (SEs) have been reported: the classical five SEA to SEE and the four additional SEG to SEJ. PCR detection of these SE genes will be carried out as described by Blaiotta et al. (2004).

10.4. Determination of hemolysin and DNase activities (CNS)

Hemolysin activity will be checked on blood agar plates (Difco) prepared with Tryptose Blood Agar Base (Becton Dickinson) and supplemented with 5% sterile defibrinated sheep blood (Biomerieux, Ca. No. 55822). The blood agar plates will be inoculated with the CNS, grown aerobically for 24 and 48 h at 37 °C, transferred to 4 °C overnight and examined for hemolytic activity according Zell et al.

(2008). Strains with hemolysis zones of more than 1 mmwidth from the border of the colony were evaluated as moderate hemolysing, strains with small zones less than 1 mm as weak hemolysing.

DNase activity will be determined on DNase Agar (OXOID CM32). CNS strains will be incubated on DNase agar at 35 °C for 18 h. After overnight incubation, 1 N HCI will be poured on the plates and colonies with clear color will be considered DNase positive.

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