| DELIVERABLE ASSESSEMENT REPORT | |
|---|---|
| | |
| **Project Title** | Preservation of micro-organisms by understanding the protective mechanisms of oligosaccharides |
| **Project Acronyme** | PREMIUM |
| **Project Number** | 777657 |
| **WORK PACKAGE** | **WP4 – Elucidation of mechanisms of LAB degradation/protection** |
| **Reference** | **D 4.1** |
| **Number** | 12 |
| **Title** | Protocol of samples handling |
| **Lead by** | INRA |
| **Editor** | MH Ropers |
| **Due date** | 30th June 2018 |

The objective of this deliverable is to summarize the different protocols of handling samples among the partners of PREMIUM in order to be shared for implementation, to ensure adequate utilization when transferring samples to other partners, thus contributing to quality of the results.

Principal type of samples include: lactic acid bacteria, mammalian cells, oligosaccharides (FOS and GOS), lipids extracts (from cells) and commercial standards of lipids and oligosaccharides. Colors help the identification of strains in both Tables and Schemes.

The document is shared into four tables and three figures (direct access to them by CTRL+CLICK on the Table/Figure):

Table 1. Protocols related to mammalian cells

Table 2. Protocols related to lactic acid bacteria

Table 3. Protocols related to lipids extracts and FOS/GOS produced in the project

Table 4. Providers of standards

**Scheme 1.** Handling protocols of Lactobacilli at INRA

**Scheme 2.** Handling protocols of Lactobacilli at CONICET

**Scheme 3.** Protocol of Biosearch for determining cell concentration (can be adapted to smaller amounts of sample)

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Table 1**. Protocols related to mammalian cells

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| **Jurkat E6.1 Cells** (Asymptote) | 10% Fetal Celf Serum in RPMI-1640 medium, supplemented with 1% fungizone and 2% penicillin | Transfer of knowledge to mammalian cells: method of preservation of mammalian cells | Use freshly thawed. Sterile culture at 37°C in a 5% CO2 humidified incubator required. | Centrifuge at 10,000 g for 4 minutes to pellet cells | Remove Cryopreservation medium from cell pellet and replace with fresh culture medium | 10% DMSO during cryopreservation. | Cryopreserved at 1°C/min, and stored at ultra-cool temperatures. | Indefinitely <-140°C, one month at -80°C, 24h at 4°C. | Use gloves during cryopreservation and thawing to prevent direct skin contact with DMSO |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Table 2.** Protocols related to lactic acid bacteria

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1** (INRA)<br><br>***Lactobacillus plantarum* WCFS1** (INRA) | Fermentor 37-42°C - MRS -Whey /lactose +Yeast extract 30°C - MRS - Glucose +Yeast extract | Stability tests/Biological activity (cultivability and acidification activity) | Yes, before freezing and also after freezing, freeze-drying or storage | 8 000 g, 15 min, 4°C | no | Protective solution: Disaccharides+ (polymer+ antioxidant); ratio 1g pellet/1g-2g protective solution; Dry matter about 25% w/w | Freezing -80°C in 1mL cryotube/eppendorf Freeze-drying: 1 mL/vial; freezing to -50°C, primary drying at -20°C and 10 Pa, secondary drying at 25°C | Days to years; | Thawing at 42°C, 5 min, centrifugation at 10000 g, 4 °C, 10min+ washing with physiological water + centrifugation 10000 g, 4 °C, 10min |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1** (INRA)<br><br>***Lactobacillus plantarum* WCFS1** (INRA) | | FT-IR | No | 8 000 g, 15 min, 4°C | no | Protective solution: Disaccharides+ (polymer+ antioxidant); ratio 1g pellet/1g-2g protective solution; dry matter about 25% w/w | Freezing -80°C in 1mL cryotube/eppendorf<br>Freeze-drying: 1 mL/vial; freezing to -50°C, primary drying at -20°C and 20 Pa, secondary drying at 25°C | Days to years; | Thawing at 42°C, 5 min, centrifugation at 10000 g, 4 °C, 10min+ washing with physiological water + centrifugation 10000 g, 4 °C, 10min |
| ***Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1** (INRA)<br><br>***Lactobacillus plantarum* WCFS1** (INRA) | | | Yes just after washing of cell pellet with physiological water | 8 000 g, 10 min, 4°C | Yes, with physiological water | Protective solution: Disaccharides, polymers, antioxidant; ratio 1gpellet/1g-2g protective solution | | | |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1** (INRA)<br><br>***Lactobacillus plantarum* WCFS1** (INRA) | | Lipid extraction and analysis (ASE + GC-MS) | no | 8 000 g, 15 min, 4°C | Yes, one to three times with physiological water or buffer Tris-HCl 50mMpH 8.8 if culture in whey based medium+ once physiological water[1] | Physiological water | Freezing -80°C in 1mL cryotube/eppendorf | Days to years | Thawing at 42°C, 5 min, centrifugation at 10000 g, 4 °C, 10min |

[1] Physiological water : NaCl 8.9 g/L

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Lactobacillus delbrueckii* subsp. *bulgaricus*** (CONICET)<br><br>***Lactobacillus plantarum*** (CONICET) | Flask<br><br>MRS broth incubation for 24 hs at 37ºC<br><br>Flask<br><br>MRS broth incubation for 24 hs at 30ºC | For storage stability tests | | 4 000 g<br>10 min | | Media used for freezing: Skim milk (12% w/v)<br><br>Media used for freeze-drying:<br>FOS (20% w/v)<br>or<br>GOS (20% w/v) | Liquid: 1 ml of bacterial suspension (approx. $10^8$ UFC/ml in freeze-drying medium), in a 5 ml glass vial, frozen in liquid nitrogen (−196 °C).<br>Dry: freeze-dried in 5 ml glass vials at 4 ºC for 48hs in a HETO FD4 lyophilizator operating with the condenser at −45 °C at a chamber pressure of 0.04 mbar. | For long term storage:<br>wet (frozen at -80ºC) in 2 ml cryovials | Reconstitute to 1 ml with 0.85% w/v NaCl. |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

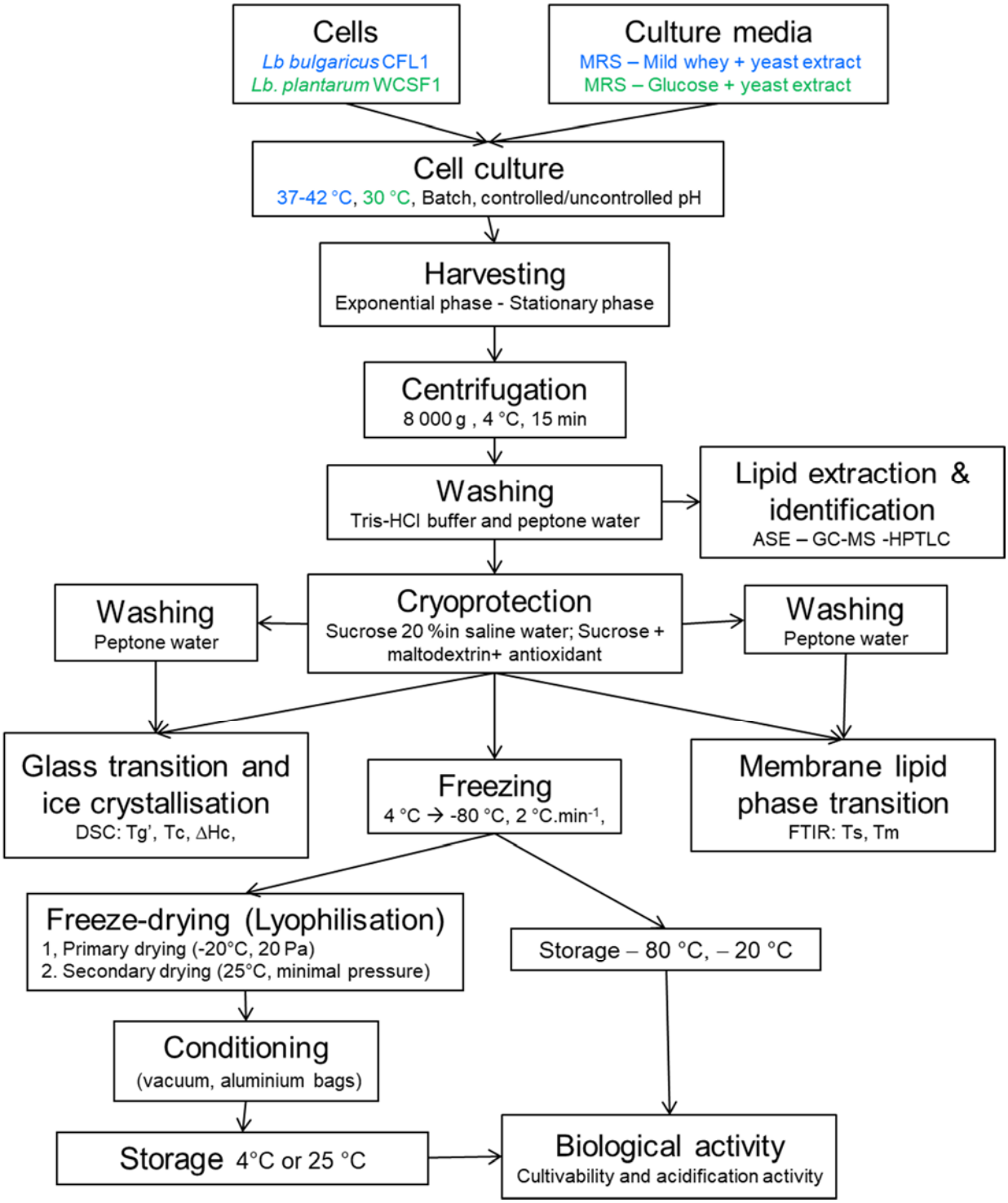
| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Carnobacterium maltaromaticum*** (Cryolog) **Working bank** *(ask Cryolog for media composition details)* | Flask MN medium | Culture T° 30°C *(OD 600nm 0.7-0.9)* | No *(Need to be Frozen at -80°C at least for a week as stabilization)* | No | No | Yes MN medium added of Glyceroled Milk in the proportion of 1 : 4 (v:v) | No drying nowadays Freezing : at least for a week at -80°C before any test | Days to years at -80°C in 1ml Cryotube | Thawing in water bath at 30°C, for 3 min Direct use *(dilution in Tryptone solution if needed)* |
| ***Carnobacterium maltaromaticum*** (Cryolog) **Biomass** *(result from fermentation of working bank culture)* *(ask Cryolog for media composition details)* | Fermentor MN medium | Biological activity (cultivability, acidification activity) | No *(Need to be Frozen at -80°C for at least a week)* | 2 635 g 10 minutes 4°C | No | Yes Centrifugation pellet diluted in Physiological Trehalose solution in the proportion of 1 : 2 (w:w) | Freezing for at least a week at -80°C No dried samples yet (to be developed) | Days to years at -80°C in different volume *(from 1ml Cryotube to 10ml container)* | Thawing in water bath at 30°C, for 3 min Direct use |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

| | | | | Treatment after production | | | | Storage | Precautions for handling |
|---|---|---|---|---|---|---|---|---|---|
| | **Culture conditions** | **Use/Measurement** | **Used freshly? Precautions? Yes/No** | **Centrifugation Speed: Time: Temperature:** | **Washing Yes/No** | **Protection Yes/No If yes, name of preservatives (sugars, anti-oxidants, polymers…)** | **Stabilization Drying: Yes/No Freezing: Yes/No If yes, T= ?°C Container:** | **Time of storage Years, Months? Temperature?** | **Rehydration/thawing procedure Temperature, time and media composition to be preferred, washing if relevant** |
| ***Lactobacillus fermentum* CECT5716** (Biosearch) | Fermentor<br><br>MRS Broth<br>37ºC<br>10-12h | Viability in MRS Agar Peptone water for vivificatio n | | 8 000 g<br>10 min<br>4ºC | Only to remove cryoprot. With peptone water | Disaccharides, antioxidant and maltodextrin | | Years @ -80ºC frozen<br>Years @ -50ºC FD Powder | Freeze-dried powder: bring to room temperature before opening vial/sachet. Thawing at 37ºC |
| ***Lactobacillus salivarius* CECT5713** (Biosearch) | Fermentor<br><br>MRS Broth<br>37ºC<br>10h | Viability in MRS Agar Peptone water for vivificatio n | | 8 000 g<br>10 min<br>4ºC | Only to remove cryoprot. With peptone water | Disaccharides, antioxidant and maltodextrin | | Years @ -80ºC<br>Years @ -50ºC FD Powder | Freeze-dried powder: bring to room temperature before opening vial/sachet. Thawing at 37ºC |

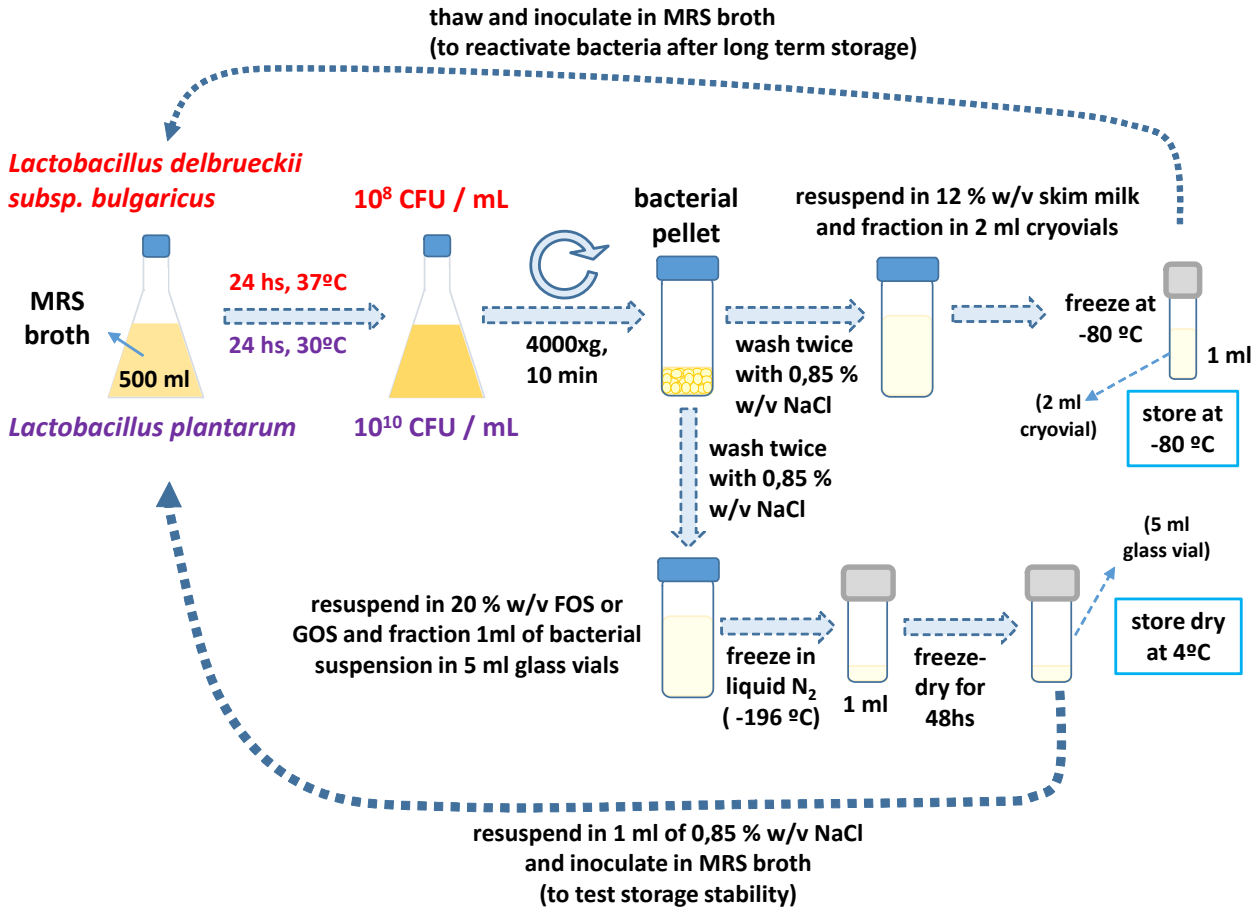
*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Scheme 1.** Handling protocols of Lactobacilli at INRA



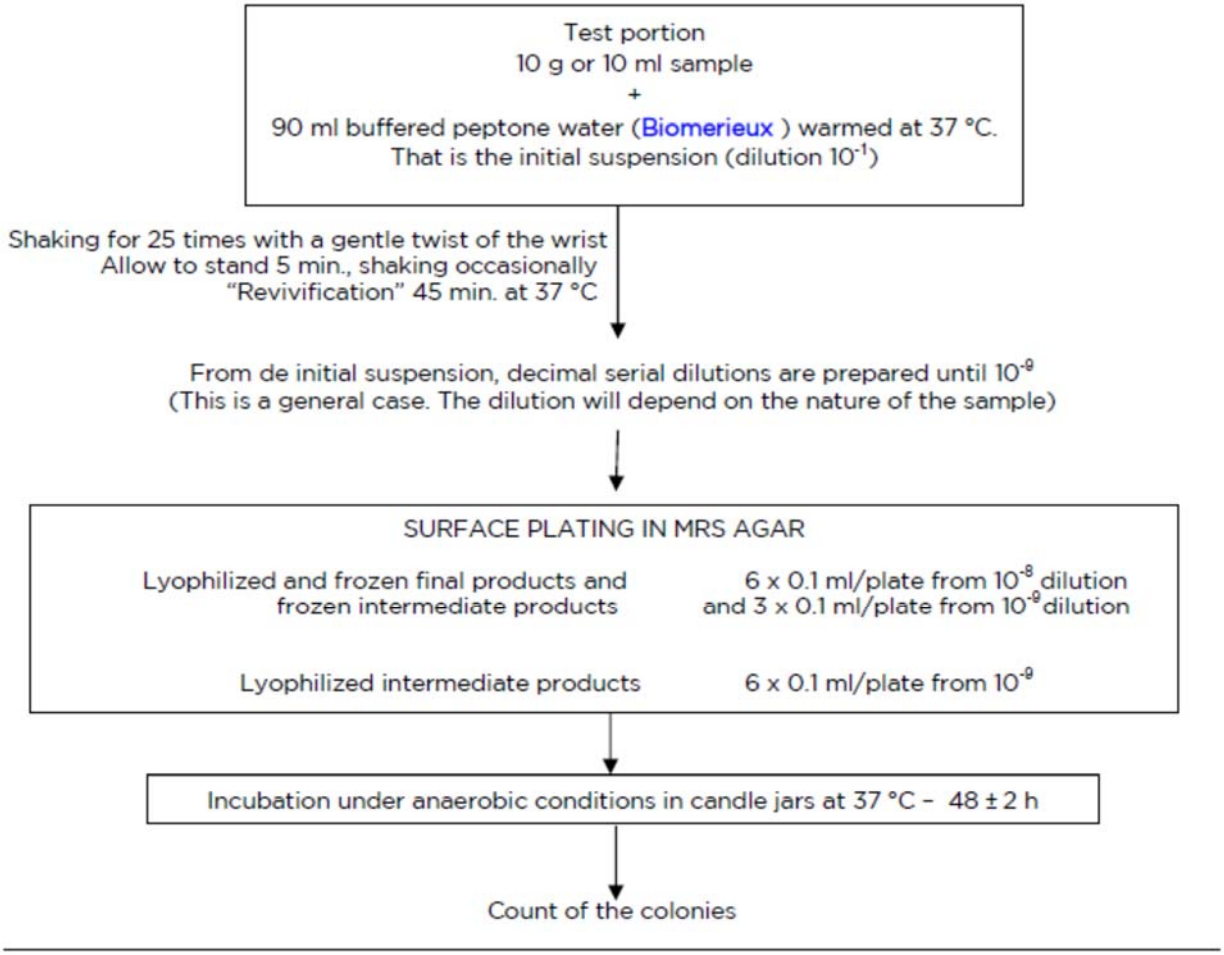

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Scheme 2.** Handling protocols of Lactobacilli at CONICET




*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Scheme 3.** Protocol of Biosearch for determining cell concentration (can be adapted to smaller amounts of sample)




| SUMMARY | |
|---|---|
| Diluent | BPT (Biomerieux) |
| Solid culture medium | MRS agar. Commercial plates (Scharlab) |
| Sample | 10.0 ± 0.1 g |
| Volume of diluent | 90 ml |
| Shaking | 25 times with a gentle twist of the wrist. Allow to stand for 5 min., shaking occasionally |
| Revivification | 45 min. at 37 °C |
| | Decimal Serial dilution until $10^{-9}$ |
| Inoculation | Surface: 0.1 ml of $10^{-8}$ and $10^{-9}$ are spread onto the surface of MRS plates (3 plates minimum of each dilution) |
| Incubation | Anaerobic conditions in anaerobic jars for 48 h - 37 °C |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

**Table 3**. Protocols related to lipids extracts and FOS/GOS produced in the project

| | Production | Storage | | | Rehydration procedure |
|---|---|---|---|---|---|
| **Samples** | **Procedures** | **Liquid or dry** | **Protective gas** | **Temperature** | |
| **Lipid extracts** | Lipids extracted from Lactic acid bacteria using *Accelerated Solvent Extraction (ASE)* and successive $CHCl_3$/MeOH mixtures. Solvents are evaporated using SpeedVac. | Dry | no | -80°C in glass vials | Physiological water/protective solution |
| **FOS & GOS** | different composition (mixtures of oligosaccharides with different DP), obtained by:<br>1) Enzymatic synthesis: from sucrose (FOS) or lactose (whey permeate) (GOS)<br>or<br>2) Hydrolysis of polysaccharides from Madeiran regional substrates (inulin from sweet potatoes; bananas; chickpea) | Liquid: in 20% w/v solution<br>Dry: freeze-dried in 2 mL cryovials (1 ml of GOS/FOS solution, dried for 24 h in a HETO lyophilizator) | no | Liquid: T =4°C °C (25°C possible)<br>Dry: at 4°C | Powder reconstituted in MilliQ water, according to the desired concentration (w/v) |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

Table 4. Providers of standards

| Samples (and/or synonyms) | Provider | Storage | Sample handling | Comments |
|---|---|---|---|---|
| DOPG<br>1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>PG(18:1(9Z)/18:1(9Z)) | Avanti Polar lipids or Sigma Aldrich* | -20°C<br>1 year | If received in the dry state : keep the vial for 20 min at room temperature, then weigh and add the expected volume of CHCl3/MeOH<br>If solubilized: used as received or diluted | INRA: preferred CHCl3/MeOH ratio 5/0.3, powder forms are preferred |
| DPPG<br>1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>1,2-dihexadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>PG(16:0/16:0) | Avanti Polar lipids or Sigma Aldrich* | -20°C<br>1 year | If dry: 20 min at room temperature, after weighing add the expected volume of CHCl3/MeOH (5/0.3)<br>If solubilized: used as received or diluted | INRA: preferred CHCl3/MeOH ratio 5/0.3, powder forms are preferred |
| POPG<br>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)<br>PG(16:0/18:1(9Z)) | Avanti Polar lipids or Sigma Aldrich* | -20°C<br>1 year | If dry: 20 min at room temperature, after weighing add the expected volume of CHCl3/MeOH (5/0.3)<br>If solubilized: used as received or diluted | INRA: preferred CHCl3/MeOH ratio 5/0.3, powder forms are preferred |
| FOS and GOS standards | FOS: Orafti p95, Beneo, Germany.<br>GOS: Bioligo GL5700, Ingredion Limited, UK | as a dry powder, at 4ºC | Reconstitute in Milli Q water, according to the desired concentration (w/v) | |

* Avanti polar lipids provide lipids with the highest degree of purity. This provider may be preferred. Sigma Aldrich is now also a provider of Avanti polar lipids products in Europe.

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*

Reminder: a material transfer agreement has to be fulfilled for each transfer from one lab to another.

| Reviewer's list | Name | Organization |
|---|---|---|
| | Yann Gohon | APT |
| | Peter Kilbride | Asymptote |
| | Julie Meneghel | Asymptote |
| | Mónica Marro | ICFO |
| | Esteban Gerbino | CONICET |
| | Sophie Keravec | Cryolog |
| | Pablo Mobili | CONICET |
| | Fonseca Fernanda | INRA |
| | Pablo Loza | ICFO |
| | Marie-Hélène Ropers | INRA |
| | Amélie Girardeau | Cryolog |
| | Andrea Gomez Zavaglia | CONICET |

*This project received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 777657*