Recovery and valorization of exhausted fermentatioN brOths and by products to reduce WASTE in the agri food sector (NO WASTE)

Elena Anna Ferrucci, DeFENS, Università degli Studi di Milano, Italy

Tutor: Prof. Stefania Arioli (DeFENS), Co-tutor: Dr. Eric Oriol (Lesaffre)

In agri-food industry, waste disposal of spent media from microbial biomasses production is a critical point. Thousands of liters of spent media are discarded, although still rich in nutrients. It implies a waste of nutrients and high environmental impact of their disposal. Among gut microorganisms, cross-feeding is known to take place with an exchange of metabolites between ‘producers’ and ‘recipients’ species. This project is aimed at recycling exhausted fermentation growth media from traditional probiotics biomass production to be used as source of purified fractions or additives to enrich industrial media needed for production of Next Generation Probiotics.

Recupero e valorizzazione di terreni di fermentazione esausti per ridurre gli scarti di produzione nel settore agro-alimentare

Nel settore agroalimentare, un punto critico della produzione industriale è lo smaltimento dei terreni esausti derivanti dalla produzione di biomasse. Migliaia di litri di terreno esausto vengono scartati, nonostante siano ancora ricchi di nutrienti. La conseguenza è uno spreco di nutrienti, il cui smaltimento ha un elevato impatto ambientale. Tra i microorganismi del microbiota intestinale avviene fenomeno di cross-feeding, cioè uno scambio di metaboliti tra specie differenti. Questo progetto è finalizzato al riciclo di terreni di fermentazione esausti, derivanti dalla produzione di probiotici tradizionali, per essere usati come fonte di frazioni purificate o additivi per l’arricchimento di terreni industriali utili per la produzione di probiotici di futura generazione.

# **1. State-of-the-Art**

In the agri-food industry, the disposal of spent media resulting from the production of microbial biomasses is a critical point. After biomass preparation and separation, thousands of liters of spent media are discarded, even though still containing high level of sugar, protein, peptides, and other nutrients, as well as organic acid and other products of the primary and secondary metabolism of the cultured microorganism (Fenster *et al*., 2019). It implies a double issue: a waste of nutrients and the high environmental impact of their disposal. Trophic interactions can occur among microorganisms of the gut microbiome, implying an exchange of nutrients so that metabolites are released as product by a species (producer) and used as nutrients by another species (recipient). As an example, species that utilize a particular polysaccharide will liberate polysaccharide breakdown products that are consumed by other species unable to grow on the polysaccharide alone. Exchanges of short-chain fatty acids (SCFAs) (e.g., acetate, propionate, and succinate), organic acids (e.g., lactate), amino acids, and vitamins are common examples of metabolic interactions (Hirmas *et al*., 2022). An interesting example are the cross-feeding interactions occurring in the gut between *Bifidobacterium* and butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, *Roseburia*, and *Eubacterium* (Lee *et al*., 2018). The latter three genera belong to the Next-generation probiotics (NPGs), promising probiotics identified through comparative bioinformatic analyses in healthy subjects. They are more difficult to be cultivated compared to traditional probiotics, since they are nutritionally demanding and highly sensitive to aerobic conditions, which translates into several technological challenges concerning large scale production (Martìn *et al*., 2019; O’Toole *et al*., 2017). Optimization of industrial media for NGPs biomass production is the focus of our project. This aim will be achieved by the valorization and re-use of exhausted fermentation broths derived from traditional probiotics biomass production.

# **2. PhD Thesis Objectives and Milestones**

The project is related to a collaboration within the University of Milan and Lesaffre, one of the largest suppliers of high-performance nutrients designed to address the diverse needs of dairy starter (e.g., lactic acid bacteria), fastidious probiotics, aerobic, and anaerobic strains for fermented food or health applications.

NO-WASTE is aimed at i) contributing to the reduction of waste and environmental impact derived from the microbial biomasses production and ii) recycling of fractions of the spent medium used for biomass production of lactic acid bacteria, considered as “producers”, to improve the growth of NGPs, considered as “recipients”.

Therefore, the main activities of the project are the following:

**A1) Deciphering the primary metabolism of producers and nutritional requirements of recipients**

Metabolic prediction-reconstruction of the primary metabolism of 7 species of lactic acid bacteria (*Streptococcus thermophilus, Lactobacillus acidophilus, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus*, *Bifidobacterium animalis* subsp. *lactis, Lactobacillus brevis* and *Lactobacillus plantarum)*; metabolic prediction-reconstruction of nutrient requirements of 4 NGPs species (*Akkermansia muciniphila, Faecalibacterium prausnitzii, Clostridium tyrobutyricum,* and *Eubacterium hallii*).

**A2)** **Biomass production of different species of traditional probiotics, for exhausted media recovery**

Biomasses of *S. thermophilus, L. acidophilus, L. paracasei, Bifidobacterium animalis* subsp. *lactis*, *L. rhamnosus, L. brevis* and *L. plantarum* will be produced at the University of Milan and Lesaffre laboratories using 1L-bioreactors.

**A3) Chemical analyses and composition of producer’s spent growth media**

*Task 1*. Spent media of producers will be analyzed to determine their composition. We will focus our attention on the quantification of specific metabolites as residual sugar, organic acids, free amino acids (or peptides, proteins, and other nitrogen sources), vitamins, and nucleotides, potential ingredients of an industrial medium for NGPs biomass production. Methods that will be used for metabolomic studies are HPLC, mass spectrometry, MALDI-TOF, NMR.

*Task 2.* Identification and purification of fractions to be used as additive for the formulation and optimization of new industrial medium for the recipient’s biomass production. Based on the composition of the spent medium, molecules will be separated through different centricon centrifugal filter devices at cut-off established based on the molecules of interest to be purified (3-100 kDa). This system will be applied for small volume of medium (1-100 ml). For a scale-up at laboratory level (up to 1 liter), macro- and micro-solutes will be separated by ultrafiltration and by Tangential Flow Filtration Membrane (TFF) (for scale-up towards higher volumes of spent media): permeate and retentate will be recovered after TFF and used as ingredient or additive for NGPs biomass production.

**A4) Enrichment-formulation of the recipient’s growth media for biomass production**

Evaluation of media enrichment on recipient’s biomass yield: specific fraction of the producer’s spent media will be added to the recipient’s growth medium at different amount. Then, different parameters will be evaluated to assess the effect of the new ingredient on the NGPs biomass yield in comparison with the in-use industrial medium.

The parameters i) final cell density and ii) viability will be evaluated by flow cytometry and by standard plate counting. Also, iii) morphology will evaluated by flow cytometry and by microscope analysis.

**A5) Process scale-up process**

After media optimization at lab scale (up to 1L-bioreactor), the scale up at industrial level will be carried out in collaboration with Lesaffre.

**A6) Data analysis, manuscripts and PhD thesis preparation**

**Table 1** *Gantt diagram for PhD thesis project.*

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| Activities  |  | Months | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 |
| A1) Deciphering the primary metabolism of producers and nutritional requirements of recipients |  |  |  |  |  |  |  |  |  |  |  |  |
| A2) Biomass production of different species of traditional probiotics, for exhausted media recovery |  |  |  |  |  |  |  |  |  |  |  |  |
| A3) Chemical analyses and composition of producer’s spent growth media |  |  |  |  |  |  |  |  |  |  |  |  |
| A4) Enrichment-formulation of the recipient’s growth media for biomass production |  |  |  |  |  |  |  |  |  |  |  |  |
| A5) Process scale-up  |  |  |  |  |  |  |  |  |  |  |  |  |
| A6) Data analysis, manuscripts and PhD thesis preparation |  |  |  |  |  |  |  |  |  |  |  |  |

# **3. Selected References**

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