Validation of Sourdough Key Players using De Novo Synthetic Microbial Communities

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This PhD thesis research project is aimed at validating the key players of sourdough using new synthetic microbial communities (SMCs). Ultimately, this project envisions the application of robust sourdough SMCs in leavened baked goods instead of starter cultures, to optimize their fermentation, resulting in essential metabolites but maintaining their functionality by overcoming the perturbations of the fermentation process.

Convalida degli attori principali della pasta madre utilizzando le comunità microbiche sintetiche de novo

Questo progetto di tesi di dottorato ha lo scopo di convalidare i principali attori della pasta madre utilizzando nuove comunità microbiche sintetiche. In definitiva, il progetto prevede l'applicazione di tali comunità nei prodotti lievitati da forno al posto delle tradizionali colture starter, per ottimizzare la fermentazione e produrre metaboliti essenziali ma al contempo conservare la loro funzionalità nonostante le perturbazioni insite alla fermentazione.

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

Microbiomes in food environments are complex, just like in the human gut. Today, steering food fermentations with starters (monocultures) alone is ecologically unsound and minimalistic. Sourdough fermentation exploits a complex microbial community (Arora *et al*., 2021). Recent studies carried out by Calabrese *et al*. (2022) revealed that a fermentome-driven sourdough fermentation (a consortium of about 8 strains) is more sustainable compared to the traditional starters. The consortium includes dominant, sub-dominant and satellite microbial players with complementary and unique metabolic functions, which ensure the stability and resilience of a mature sourdough. Sourdough microbial communities are typically composed of lactic acid bacteria such as *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Pediococcus pentosaceus* and *Furfurilactobacillus rossiae*, as well as yeast species such as *Saccharomyces cerevisiae* (Gobbetti *et al*., 2014). Metaomics analyses have shown that these communities may produce various metabolites that may have beneficial effects on the human gut microbiome, including short-chain fatty acids and antimicrobial peptides (Da Ros *et al*., 2021).

Mechanistic fermentation studies are complicated since observed functionalities result from diverse metacommunities rather than single species (Friedman *et al*., 2017). Coupled with metaomics, one of the complexity-reducing strategies developed to explain not only the compositional characteristics, but also the mechanistic causation influencing the emergent structural and functional traits of microbial communities is the use of Synthetic Microbial Communities (SMCs) (Calabrese *et al*., 2022; Karkaria *et al*., 2021). The SMCs approach is an emerging technique that involves co-culturing multiple taxa under well-defined conditions to mimic the structure and function of a microbiome. The metaomics approach has emerged as a powerful tool for studying complex and dynamic microbial consortia. It involves the simultaneous analysis of multiple types of biomolecules (DNA, RNA, proteins, and metabolites) to gain a comprehensive understanding of the functional and structural properties of microbial communities.

Overall, the current state-of-the-art reveal the need for further research to fully understand the mechanistic processes of sourdough microbiome. The recent study (Calabrese *et al*., 2022) did not reveal whether the robustness of sourdough metacommunities is strain- or species-specific. Therefore, this research seeks to validate the key players of sourdough using new SMCs composed of substitute strains and species. Overall, this study will change the paradigm and introduce theoretical foundations for guiding food fermentations.

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

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review and preliminary work**: the first months of the PhD have been dedicated to review of the state-of-the-art relevant to this thesis (A1.1). Pure isolates of the bacteria and yeast strains needed for the SMC construction will be obtained and confirmed after DNA extraction, standard PCR and Sanger’s sequencing (A1.2).

A2) **Construction of new sourdough synthetic microbial communities**:Wheat flour hydrolysate (WFH) will act as the model medium (A2.1). Autochthonous strains isolated from sourdoughs all around the world will be used. A total of eighteen (18) SMCs will be constructed in WFH (A2.2). This will comprise of new species/strains substituting the dominant, sub-dominant and satellite species/strains as defined by the prior research of Calabrese *et al*. (2022).

A3) **Competition experiments between dominant and sub-dominant lactic acid bacteria**:members from the most performing SMCs from M2 will be evaluated in pairs (one dominant with one sub-dominant) for specific interspecies competition assays. One dominant and one sub-dominant will be inoculated at 107 cfu/ml each in WFH, growth kinetics will establish the end of exponential phase. One per cent of the co-culture at the exponential phase of growth will be inoculated again in WFH and growth kinetics will be monitored for the total of 5 days (Janßen *et al*., 2018) (A3.1). Quantitative PCR (qPCR) will be used to determine the absolute abundances of the species during the interactions, metatranscriptomics and metabolomic analyses will be used to evaluate the metabolic interaction of the selected dominant and sub-dominant strains. Total number of all possible interactions for the members of the most performing new SMCs will be 6 and time points will be 3 (0, 2 and 5 days). Total number of samples will be 18 and analysed in duplicates (A3.2).

A4) ***In situ* experiment for stability and robustness of new SMCs**:to complete the design and validate the approach explained in A1 and A2, the most performing SMCs will be also evaluated under *in situ* conditions. Strains will be inoculated according to their abundance classification (core dominant, core sub-dominant, etc.) in specific cell densities in water and flour and will be fermented according to the conditions established for SDGlobal (A4.1). After maturation, the new SMC sourdough will be daily propagated for 30 days and each 10 days samples will be evaluated for the persistence of strains and their cell densities using qPCR. The same approach will be used for the most performing SMC that included *Kazachstania humilis* without *S. cerevisiae*. The goal is to evaluate if S. cerevisiae will find its way to appear during the daily propagations and consequently dominate over *K. humilis* (A4.2).

A5) **Research publications and thesis**: writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

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

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Activity Months | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| A1) | ***Preliminary Work***  |   |   |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |
|  | 1) State-of-the-Art Review |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |
|  | 2) Pure Culture Isolation |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |
| A2) | ***New Sourdough SMCs Construction*** |   |   |   |  |  |  |  |   |   |  |  |  |  |   |   |   |  |   |   |   |  |  |  |  |
|  | 1) Preparation of WFH Media |   |   |   |  |  |  |  |   |   |  |  |  |  |   |   |  |  |  |   |   |  |  |  |  |
|  | 2) SMCs Construction in WFH |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |   |  |   |   |   |  |  |  |  |
| A3) | ***Competition Experiments*** |   |  |   |   |   |   |  |   |   |   |   |   |  |   |  |  |  |  |   |   |   |   |   |  |
|  | 1) SMCs Interspecies Competition |   |  |   |   |   |  |  |   |   |   |   |  |  |   |  |  |  |  |   |   |   |   |  |  |
|  | 2) qPCR and Meta-Omics Analyses |   |  |   |  |  |   |  |   |   |  |  |   |  |   |  |  |  |  |   |   |  |  |   |  |
| A4) | ***In Situ Experiments*** |   |  |   |  |  |   |   |   |   |  |  |   |   |   |  |  |  |  |   |   |  |  |   |   |
|  | 1) SMCs *In Situ* Assay |   |  |   |  |  |   |   |   |   |  |  |   |   |   |  |  |  |  |   |   |  |  |   |   |
|  | 2) Stability and Robustness Analyses |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |
| A5) | ***Research Publication and Thesis*** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |   |   |   |   |   |   |   |

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