ONE EARTH

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Table of Contents

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List of Tables

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List of Figures

List of Abbreviations and Acronyms	
PUFA	Polyunsaturated fatty acids
CW	Cheese Whey
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
VFAs	Volatile fatty acids
SCO	Single Cell Oil
YPD	Yeast extract, Peptone, Dextrose
YNB	Yeast nitrogen base
BDL	Below detection limit
FFA	Free fatty acids
FA	Fatty acids
OD	Optical density
SCFA	Short chain fatty acids
TRL	Technology Readiness Level
RT	Room temperature
LT	Low temperature
OD_{600nm}	Optical density at 600 nm
HPLC	High-performance liquid chromatography
CDW	Cell dry weight
EFT	Estimated fermentation time

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1 Executive Summary

This deliverable presents the results of Subtask 2.2.1, which aimed to evaluate the production of polyunsaturated fatty acids (PUFAs) and single cell oils (SCOs) from volatile fatty acid (VFA)-rich effluents using two microbial platforms: marine bacteria and the oleaginous yeast *Yarrowia lipolytica*. The activity was conducted at TRL 3 by UNIBO and UNIPR, in collaboration with UNIVPM and Biotrend.

Marine strains previously isolated by UNIBO were studied for their capability of accumulating PUFAs, with particular attention to the production of target Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. After unsatisfactory results were obtained, a deep search for alternative bacterial strains potentially able to produce PUFAs was conducted by three approaches: a bibliographic search was carried out by screening recently published scientific articles; a bioinformatic study was applied with the aim of identifying bacteria with recognized genetic makeups for the production of PUFAs; and a screening of bacteria available in a microbial collection of UNIPR was conducted by performing a laboratory test determining the bacterial resistance to an oxidative stress, which can be associated to the occurrence of PUFAs within bacterial membranes. However, just three strains that were purchased from commercial collections in agreement with the literature review and the bioinformatic study gave rise to promising evidence when grown in culture media suggested by suppliers, namely, *Shewanella pacifica, Shewanella frigidimarina* and *Moritella marina*. In particular, both *Shewanella* strain were observed to produce EPA, while the *Moritella* sp. appeared to be able to accumulate DHA. Thus, *Shewanella* strains were chosen as the biocatalysers for next experimental steps, concerning the employment of VFAs as the feedstock, in agreement with project aims. Both strains were cultivated in a 6 L-fermenter following a two-step procedure, respectively dedicated to allow microorganisms to colonize the reactor under more favourable conditions by adding glucose to the conventional medium, and, once glucose is completely depleted, to feed grown bacteria with a mixture of commercial VFAs in order to induce them to convert short chain acids into target PUFAs. Importantly, both organisms were observed to produce EPA: in particular, *S. pacifica* and *frigidimarina* accumulated about 3.6% and 0.9%, respectively, of EPA within the overall lipidic fraction; however, the former culture was poorer in lipids (about 2% and 10%, respectively, of total solids, including ashes, whose amount is typically improved by the occurrence of undissolved salts). Basing on those data, *S. frigidimarina* was then cultured under different conditions, in order to optimize the composition of the culture medium. In particular, since salts precipitation was observed in the preparation of the solution, Fe-citrate was replaced by Fe-EDTA, which allowed to obtain a clear medium containing all the components required for microbial growth. Thereafter, one experiment was set up for abating the content of peptone and yeast extract in the medium, this resulting in limiting the availability of C sources alternative to VFAs, with the aim of enhancing VFA uptake for microbial growth. Results suggest that the addition of yeast extract can be avoided, while comparable PUFA production was observed by reducing peptone concentration of one order of magnitude. A further experiment dedicated to optimizing the composition of the culture medium demonstrated that effective PUFA productions can be obtained by adopting one modified mineral medium including only 50 % of the salts concentration. Finally, one test was conducted to demonstrate the feasibility of feeding the bacteria with an actual VFA rich-fermentation broth rich in VFAs, which was obtained by fermenting cheese whey under anaerobic acidogenic conditions. Importantly, 1.06% of EPA on the overall lipidic fraction was obtained, representing the latter fraction more than 12% of the overall solids.

Based on UNIBO's inputs on the bacteria fermentation development in small scale, three bacteria strains were selected to be tested by Biotrend: *Moritella marina*, *Shewanella frigidimarina* and *Shewanella pacifica*. After a screening test in shake flasks scale, only *S. frigidimarina* was able to growth on Biotrend's fermentation conditions being the only bacteria selected to start the data collection about the bacteria fermentation dynamics in view of lab-scale fermentations. Since the rich medium used by Biotrend has a different composition from the culture medium used by UNIBO, a shake-flasks medium screening test was done with both media to evaluate their impact on bacteria biomass and PUFAs production and composition. In this growth study, a higher biomass production was observed using Biotrend's rich medium. At the time of this deliverable submission, no PUFAs production and composition results are presented, since this analysis is still ongoing.

In parallel, ten wild-type strains of *Y. lipolytica* were initially screened in minimal Yeast Nitrogen Base (YNB) media supplemented with increasing concentrations of acetic acid (0.2–1%). Growth monitoring over 72 hours allowed the selection of three representative strains (Y3, RO2, RO3.) with distinct tolerance and metabolic responses. These strains were subsequently tested in VFA-enriched YNB media and, later, in VFA-enriched whey provided by UNIVPM. Experiments were carried out at two different temperatures (Low or Room Temperature), under static and stirred conditions, and over variable incubation periods (3–20 days). Biomass yield, lipid accumulation, and fatty acid profiles were systematically assessed. Growth at LT for 20 days would improve the relative abundance of (P)UFA but the final yield is below the 1 % (fat/ dry biomass) with respect to the growth at RT for 13 days where the yield was above 2%, based on the strain applied. In fact, *Y. lipolytica* Y3 resulted the most promising with a 11.5% fat/dry biomass, where C18:1, C18:2, C18:3 together accounted for the 38-46% of total fatty acids. No PUFAS were observed if not in very low amount (<0.4%). The strain was further tested in a not optimised Squacquerone whey anaerobically fermented for 5 days by UNIVPM. The trial was performed for 3 and 13 days at RT in static and stirring condition. The longer the incubation the higher amount of fat was produced. Finally, Y3 was grown for 3 days in static or stirring conditions, at RT in squacquerone-whey enriched in VFAs, with and without *Propionibacterium*, produced by UNIVPM (see D2.1). The better yield was obtained with natural-fermented whey which provided 5.7 g/L of dry biomass with 2.7 % of fat/ CDW composed of C18:2 (24–29%), C18:1 (24–30%), C16:1 (13–23%), C17:1 (6–8%) (UFA: 82-85%).

Meanwhile, two *Y. lipolytica* strains (RO3 and Y3) were sent by UNIBO to Biotrend, to start the data collection for fermentation dynamics characterization in view of lab-scale 2 L fermentations preparation. A shake flask pre-test with RO3 strain was performed using a modified YPD medium (with lower nitrogen source concentration), supplemented with glucose or glycerol as initial carbon sources and, pulses of commercial VFAs solutions (acetic, butyric or propionic acids) were added to the medium during the fermentation. This yeast strain was able to grow on all the fermentation conditions tested (5.85–11.85 g/L of CDW) and the following VFAs consumption capacity was observed: propionic < butyric < acetic. Low fatty acids (FAs) content on yeast biomass was observed (1-2 % of FA/CDW), mainly composed of C16:0, C16:1 and C18:1. The result obtained with RO3 strain showed the robustness of *Y. lipolyitica* fermentation process, since similar FA production results were attained using different fermentation conditions.

Overall, Subtask 2.2.1 demonstrated the feasibility of converting VFA-rich effluents into microbial lipids, providing a proof-of-concept at TRL 3. However, for what concerns *Y. lipolytica*, no PUFAs were obtained. The optimized strains and process conditions identified herein both in minimal medium and

cheese whey enriched in VFAs will form the basis for subsequent pilot-scale validation (Subtask 2.2.2) within the ONE-EARTH project, contributing to the sustainable bioconversion of agro-industrial residues into high-value bioproducts.

2 Context and Objective of PUFAs and SCO production

Marine bacteria as well as oleaginous yeasts such as *Yarrowia lipolytica* are increasingly recognised as promising microbial platforms for the valorisation of agro-industrial residues into high-value products, notably, PUFAs [1] and single cell oils (SCOs) [2]. These microbial lipids are considered sustainable alternatives to plant and fish oils, with applications spanning food, feed, nutraceuticals, and bio-based materials.

A particularly attractive carbon source for these processes is represented by volatile fatty acids (VFAs), which can be obtained from the anaerobic fermentation of organic wastes [3]. VFAs—including acetic, propionic, and butyric acids—are low-cost intermediates that can be metabolised for instance by *Y. lipolytica* to accumulate lipids rich in unsaturated fatty acids [4,5]. Their use not only contributes to circular economy strategies but also reduces reliance on conventional sugar-based feedstocks.

Cheese whey-derived VFAs are especially suitable in this context, as demonstrated in Subtask 2.1.1, where acidogenic fermentation enabled the conversion of dairy residues into VFA-rich effluents. Building on these results, Subtask 2.2.1 explored the ability of marina bacteria, among which, *Shewanella frigidimarina*, and *Y. lipolytica* strains to grow on VFAs to accumulate PUFAs and SCOs under controlled conditions.

For both bacteria and yeasts, the experimental strategy was structured in progressive phases, first applying minimal media including VFAs, alone or in a mix, and then real substrates, meaning fermented cheese whey enriched in VFAs, mainly provided by UNIVPM. All these phases were useful to select the proper strain to apply in this project and the main growth conditions which can affect fatty acid accumulation and composition.

These activities were carried out within WP2 – Upgrading of fermentation strategies for tailored VFA production and conversion, and specifically under Task 2.2 – PUFA production from VFAs. The following sections detail the methodology applied, the experimental configurations tested, and the main results obtained, linking them to the objectives of WP2 and outlining the next steps for optimisation and pilot-scale validation.

3 Aim of the Study

This study was carried out within Task 2.2 – PUFA production from VFAs, specifically addressing Subtask 2.2.1, which aims to evaluate the production of polyunsaturated fatty acids (PUFAs) and single cell oils (SCOs) from volatile fatty acid (VFA)-rich effluents using two microbial platforms: marine bacteria and the oleaginous yeast *Y. lipolytica*.

The specific objectives of the study were:

1. To define the target bacterial and *Y. lipolytica* strains capable of growing on VFAs;

2. To define best conditions for the growth of tested bacteria and *Y. lipolytica* for optimizing the obtainment of fats with high unsaturation degree at the lab-scale;
3. To provide guidelines for pilot-scale production of (P)UFA.

4 Materials and Methods

This section describes the experimental setup and procedures adopted for the optimization of fats production with *Y. lipolytica* and bacteria using VFA as substrates. The section is structured as follows: sections from 4.1 to 4.5 refer to activities carried out with the yeast; sections from 4.6 to 4.10 refer to activities carried out by employing bacteria as PUFA producer organisms.

- Section 4.1: Screening the growth of 10 strains of *Y. lipolytica* in minimal medium enriched in commercial VFAs at different concentrations.
- Section 4.2: Test the growth and fat production of 3 strains of *Y. lipolytica* in minimal medium enriched with a mix of VFAs modulating growth temperature (cold and room temperature) and time (20 and 13 days, respectively).
- Section 4.3: Test the growth and fat production of *Y. lipolytica* Y3 in squacquerone enriched in VFAs provided by UNIVPM modulating growth condition (static agitation) and time (3 and 13 days)
- Section 4.4: Test the growth and fat production of *Y. lipolytica* Y3 in squacquerone enriched in VFAs (produced with and without Propionibacterium) provided by UNIVPM modulating growth condition (static agitation) for 3 days.
- Section 4.5: Test the growth and PUFAs production of *Y. lipolytica* RO3 strain using a modified YPD medium supplemented with glucose or glycerol and, pulses of commercial VFAs solutions (acetic, butyric and propionic acids) were added during the fermentation.
- Section 4.6: Selection of bacteria potentially capable of producing target PUFAs.
- Section 4.7 Screening the growth of selected bacteria in mineral media to verify their ability of producing target PUFAs.
- Section 4.8: Test the growth of selected *Shewanella* spp. in minimal media enriched in commercial VFAs by a bench-scale fermenter.
- Section 4.9: Test the growth of selected *Shewanella frigidamarina* in a mineral medium enriched in VFA-rich fermented cheese whey
- Section 4.10: Optimization of the mineral medium to be employed for the accumulation of target PUFAs by the selected *Shewanella frigidimarina* strain.
- Section 4.11: Growth screening test using three different marine bacteria in rich medium and glucose as carbon source.
- Section 4.12: Test the growth and PUFAs production of *S. frigidimarina* DSM 12253 in two different media, rich media and mineral medium, using with glucose as carbon source.

4.1 Screening the growth of 10 strains of *Y. lipolytica* in minimal medium enriched in commercial VFAs

The 10 strains were previously selected based on the capability to grow well in cheese whey [2]. The strains selected were: RO25, Y3, RO3., PO2, PO1, RO2, PO10, RO21, PO19, and Y22A, all belonging to the culture collection of the Department of Agricultural and Food Sciences (DISTAL), UNIBO. After culturing in Yeast extract, Peptone, Dextorse (YPD) broth, a minimal Yeast Nitrogen Base (YNB) medium supplemented with amino acids and 0.2% (w/v) glucose was prepared. This formulation aimed to establish in *Y. lipolytica* cells a "starvation–survival" condition, leading to metabolic arrest and basal activity. The ten wild-type strains of *Y. lipolytica* were inoculated in liquid YNB-glucose medium contained in test tubes and subjected to sequential transfers in order to ensure physiological adaptation. Subsequently, a minimal YNB medium containing 0.2% (v/v) acetic acid was prepared and adjusted to pH 6.8. For each of the ten strains, 10 mL of this medium was dispensed into sterile test tubes and inoculated with 100 µL of microbial culture previously grown in YNB supplemented with 0.2% glucose. Tubes were incubated for 72 h, after which visible microbial growth was observed.

To follow the growth kinetic in VFA, YNB medium was prepared with increasing concentrations of acetic acid, propionic acid and butyric acid: from 0.1% to 1% (v/v) as suggested by Llamas et al. [4].

A 96-well microplate was used to evaluate yeast growth under the different VFA concentrations. Each well was inoculated with 50 µL of a preadapted microbial culture and 250 µL of the corresponding VFA-supplemented medium, resulting in a final working volume of 300 µL per well. The microplate was placed in a Tecan Spark spectrophotometer, which automatically measured optical density at 600 nm (OD600) every hour throughout the incubation period. Growth kinetics were derived from the OD600 profiles of each strain obtained with the Tecan Spark (Fig. 1), allowing comparison of growth performance and selection of the most robust strains. Based on these results, three strains (Y3, RO3., and RO2) were selected for further experimentation.

Figure 1. Tecan Spark used to follow yeast kinetic growth curve.

4.2 Test the growth and fat production of 3 strains of *Y. lipolytica* in minimal medium enriched with a mix of VFAs

To evaluate the ability of *Y. lipolytica* strains Y3, RO3., and RO2 to grow and accumulate lipids in the presence of a mix of VFAs, YNB medium was supplemented with acetic, propionic, butyric, valeric, isovaleric, and caproic acids at the concentrations reported by Llamas et al. [4].

Six sterile flasks containing 300 ml of the VFA-enriched YNB each were prepared for each strain. For each strain, half of the flasks were incubated at room temperature, while the other three were incubated in lower temperature. Based on literature data and increased absorbance measured over time, samples were collected after 13 and 20 days for the two temperatures tested, respectively for microbial and fat characterization.

Dry matter content was determined to evaluate biomass production of *Y. lipolytica* by placing 25 ml of each culture into pre-weighed aluminium pans. Samples were dried at 60 °C until constant weight. Biomass dry matter was calculated as the difference between the final and initial weights. Lipids were extracted using a modified Folch protocol, dried, and recovered as total lipid fraction. Extracted fatty acids were derivatized to fatty acid methyl esters (FAMEs) with KOH-Methanol and analysed by GC–MS (Fig. 2) for yield and compositional profiling.

Figure 2: GC-MS (Agilent Technolgies) applied to measure fat composition

4.3 Test the growth and fat production of *Y. lipolytica* Y3 in squacquerone enriched in VFAs provided by UNIVPM

At the beginning of December 2024 UNIVPM provided DISTAL-UNIBO with 4 different cheese whey with potential VFA, namely Ricotta-2d; Ricotta-5d; Squac-2d; Squac-5d. The cheese whey was post-treated by centrifugation and subsequent microfiltration with a 0.45-micron filter. From VFA quantification performed by DICAM-UNIBO, the only sample with the highest acetic acid content was Squac-5d with 0.71 g/L. Therefore this substrate was applied to assess the fat production by Y. lipolytica Y3. The culture conditions tested were static or stirring conditions for 3 and 13 days at room temperature. At these timepoint samples were collected and analysed for dry biomass and lipid

content/composition as described in section 4.2. In particular, for lipids 50 ml of the fermented whey incubated with *Y. lipolytica* was used.

4.4 Test the growth and fat production of *Y. lipolytica* Y3 in squacquerone enriched in VFAs (produced with and without *Propionibacterium*) provided by UNIVPM or with Kefir grains by DISTAL-UNIBO

On June 2025 UNIVPM provided DISTAL-UNIBO with two new batched of fermented whey, one naturally fermented (natural-fermented whey) and one fermented with the addition of the commercial *Propionibacterium freudenreichii* (PP-fermented whey). Samples were centrifuged, filtered with 0.45 and 0.2 µm. In parallel, DISTAL-UNIBO produced a fermented whey by applying Kefir grains. The three substrates were inoculated with Y. lipolytica Y3 and incubated at room temperature for 3 days. VFAs were characterised at the beginning and end of fermentation through Solid phase microextraction (SPME) combined with GC-/MS. Lipid content, biomass production and cell growth were determined as described in the previous chapters.

4.5 Test the growth and PUFAs production of *Y. lipolytica* RO3 strain using a modified YPD medium supplemented with glucose or glycerol and pulses of commercial VFAs solutions

On February 2025, UNIBO sent to Biotrend two *Y. lipolytica* strains (Figure 3), RO3 and Y3, selected from the yeast strain screening activities. The two strains were reactivated, and internal cell banks were prepared using YPD medium. For this activity, 25 mL shake flasks cultivations were performed, and the cultures were incubated at 30ºC with 150 rpm of agitation. The reactivated cultures were cryopreserved at -80ºC.

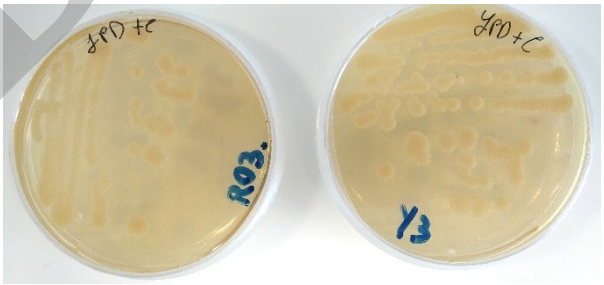

Figure 3 – Y. lipolytica strains RO3 and Y3 sent by UNIBO to Biotrend.

The data collection for fermentation dynamics characterization started with a shake flasks experiment with RO3 yeast strain. For this experiment, the cryopreserved yeast was inoculated in 25 mL modified YPD medium with lower nitrogen source concentration (yeast extract and peptone) to enhance the FA production. The initial medium was supplemented with glucose or glycerol and, a 2 g/L commercial

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VFA solution pulse was added to the medium when a slowdown on yeast growth was observed. For this experiment, 6 different medium conditions were tested:

- Test 1 – Medium with 20 g/L glucose + Pulse 2 g/L glucose
- Test 2 – Medium with 20 g/L glucose + Pulse 2 g/L acetic acid
- Test 3 – Medium with 20 g/L glucose + Pulse 2 g/L propionic acid
- Test 4 – Medium with 20 g/L glucose + Pulse 2 g/L butyric acid
- Test 5 – Medium with 20 g/L glycerol + Pulse 2 g/L glycerol
- Test 6 – Medium with 40 g/L glucose

All the cultivation tests were performed at 30ºC with 150 rpm of agitation, during 96 h.

In these experiments, yeast growth was monitored by optical density measurement at 600 nm (OD_{600nm}) and biomass production was quantified gravimetrically. Glucose, glycerol, acetic acid, butyric acid and propionic acid were quantified by HPLC analysis. FA content in biomass and lipids profile were quantified by FAMES analysis.

4.6 Selection of bacteria potentially capable of producing target PUFAs.

The selection of bacterial strains capable of producing target PUFAs by employing VFAs as the main carbon source was carried out in agreement with Task 2.2 aims. To this aim, the following approach were followed:

- 4.6.1 UNIBO performed an exhaustive bibliographic search by screening a wide range of recent publications, available on main scientific databases such as Scopus and Web of Science (WoS) [6-47], in order to define a list of commercially available strains capable of producing target PUFAS;
- 4.6.2. UNIBO and UNIPR screened three marine bacteria belonging to one UNIBO collection (G16.20, P11.20 and P16.20, respectively characterized as *Marinobacter similis*, *Halomonas titanicae* and *Marinobacter algicola* strains [15]) by inoculating and cultivating them in triplicate in 500 mL-shake flasks (150 mL of working volume), by using MB514 (suggested by DSMZ as a feasible medium for supporting the growth of marine bacteria) as the experimental medium amended with Glucose (6 g/L).
- 4.6.3 UNIPR evaluated the potential capability of producing target PUFAs in silico in *Shewanella pacifica, Shewanella frigidimarina and Moritella marina* species, which were chosen as promising PUFA producers among those evidenced by the literature search. Specifically, the presence of the five proteins involved in PUFA production (pfaA-E) [1] was assessed through research on NCBI databank (https://www.ncbi.nlm.nih.gov/), directly or through alignment performed with Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against proteins belonging to phylogenetically cognate microorganisms (*Shewanella* sp., *Shewanella pneupatophori* or *Moritella viscosa* were used in this study);
- 4.6.4 UNIPR performed a screening on 23 microorganisms belonging to the University of Parma Culture Collection (UPCCO), which were selected on the bases of the exhaustive literature review mentioned above (Section 4.6.1); the screening test is based on the capability of the PUFA producing-bacteria to be resistant to the oxidative action of hydrogen

peroxide (H_2O_2) [49]. Briefly, microorganisms were spread on agar plates on which filter paper discs will be placed and soaked with different concentrations of H_2O_2 (0.1%, 0.5% and 1%). The plates were incubated at the optimal temperature of each microorganism until full growth. Due to the membrane-shielding effects of PUFAs [48], PUFA-producing microorganisms should be able to grow around the disks, whereas a clear inhibition halo is expected for non-producing strains. More in details, screened bacteria belonged to *Lactococcus lactis* species (5 strains), *Lactiplantibacillus plantarum* species (5 strains), *Lacticaseibacillus rhamnosus*, *Latilactobacillus curvatus*, *Pediococcus acidilactici*, *Planomicrobium* genera (3 strains), *Paracoccus* genera (3 strains), *Luteimonas* genera, *Agrococcus* genera, and *Brachybacterium* genera. Media used were MRS (VWR Chemicals) for the lactic acid bacteria growth, M17 (Millipore) for the growth of Lactococcus lactis strains and TSB (Oxoid) for the growth of the other microorganisms. Each medium was amended with 1.5 g/L agar and 1 mM NaN3 as catalase inhibitor.

All PUFA analyses were carried out by the approach described elsewhere [50].

4.7 Screening the growth of selected bacteria to verify their ability of producing target PUFAs

According to evidence derived from the selection activity mentioned above (Section 4.6), UNIBO and Biotrend screened the three bacterial strains *Moritella marina* DSM 104096, *S. frigidimarina* DSM 12253 and *S. pacifica* DSM 15445, which were purchased from the DSM commercial collection. UNIBO carried out the experiments under same conditions, which were employed for screening bacteria belonging to the UNIBO collection (Section 4.6.2). In particular, the three strains were incubated in 500 mL flasks filled with 150 mL of the marine broth medium MB514, which was amended with 6 g/L of glucose to promote PUFA accumulation. Standard culture conditions (medium, pH and temperatures) were applied as indicated by the DSM supplier to pre-grow microorganisms.

Besides, Biotrend grew the strains using a rich medium in 25 mL shake flask cultivations. The three bacteria cultures were incubated at 20ºC and 150 rpm of agitation, during 72 h EFT. Bacteria growth was monitored by optical density measurement at 600 nm (OD_{600nm}) and glucose was quantified by HPLC analysis.

According to the obtained results, Biotrend carried out a last experiment with the bacteria *S. frigidimarina* DSM 12253. Since Biotrend used a different medium used by UNIBO, rich media and marine broth 2216 (MB514) were tested to evaluate the impact of each medium on biomass and PUFAs production. Thus, a 25 mL shake flask test was performed. Bacteria were incubated at 20ºC and 150 rpm of agitation, during 168 h EFT. During the growth study, bacteria growth was monitored by OD_{600nm} measurement, and the final biomass production was quantified gravimetrically (CDW). Glucose was quantified by HPLC analysis. Fatty acids content in biomass and lipids profile are being quantified by FAMES analysis.

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4.8 Test the growth of selected *Shewanella* spp. in mineral media enriched in commercial VFAs by a bench-scale fermenter

After assessment of PUFAs production through GC-MS on a small scale, *Shewanella pacifica* DSM 15445 and *Shewanella frigidimarina* DSM 12253 were grown in a Solaris model bioreactor (Solaris Biotech, Porto Mantovano, Italy) to obtain cellular biomasses. The microorganisms were cultured in 1.1L and 4 L, respectively, of culture medium composed as reported in the following Table 1:

Table 1. Composition of the mineral medium employed in the fermentation experiments carried out in a bench scale 6 L-bioreactor

	Component	**Value**	**Unit**
Solution 1	NH_4Cl	0.8/2.5	g/L
	NaCl	24.4	g/L
	Na_2SO_4	3.25	g/L
	KCl	0.55	g/L
	$NaHCO_3$	0.16	g/L
	KBr	0.08	g/L
	Na_2HPO_4	0.27	g/L
Glucose solution	Glucose	0.5	g/L
Solution 2	$MgCl_2x6H_2O$	0.5	g/L
Solution 3	$CaCl_2x2H_2O$	0.01	g/L
Solution 4	Trace element solution	10	mL/L
Solution 5	Fe (III) citrate	0.05	g/L

After the glucose consumption, the microorganisms were feed with 0.3 g/L VFAs doses (17% acetic acid, 23% propionic acid, 60% butyric acid). Each dose was supplied after the consumption of the previews one. The cultures were grown in aerobiosis at 25°C for 9 days, OD_{600} was measured at different time points and pH was maintained between 7.3 and 7.6.

4.9 Optimization of the mineral medium to be employed for the accumulation of target PUFAs by the selected *Shewanella frigidimarina* strain

After the marine bacterium *Shewanella frigidimarina* was chosen as the bacterium to be exploited for the development of the project PUFA value chain, two sets of experiments were carried out for i) optimizing the composition of the medium, and ii) verifying that the *Shewanella frigidimarina* can grow and accumulate PUFAs when fed with actual fermentation broths rich in VFAs (Section 4.10).

The optimization of the mineral medium was addressed to avoid the precipitation of salts, which was observed in previous experiments, and which results in an unproper consumption of chemicals and in an enhanced amount of inorganic matter included in the biomass collected after fermentation processes, this leading in lowered concentration of PUFAs and organic components occurring in the raw material to be used for the formulation of end products (e.g., fish feed); besides, a study was

dedicated to verify if the employment of peptone and yeast extract medium components, which contain undefined carbon sources that can be preferentially taken up by microorganisms instead of target VFAs, can be lowered or avoided, this leading to a limited requirement of chemicals, too. To those aims, the following tests were carried out:

- Fe-citrate replacement: a mineral medium containing Fe nutrient including in the complex Fe-EDTA molecule (0.1 g/L, this leading to an equivalent concentration of Fe with respect that available by the Fe-citrate compound in MB514) was tested, in order to avoid the employment of Fe-citrate, which was observed to precipitate in the medium solution. Three conditions were set up and conducted in triplicate: two with Fe-EDTA (one with and one without Peptone and yeast extract) and one with Fe-citrate (without Peptone and yeast extract);
- optimization of peptone and yeast extract contents: after the previous experiment evidenced that the removal of both peptone and yeast extract from medium components don't allow microbes to grow properly, a screening test was conducted in 500 mL shake flask (150 mL of mineral medium + glucose 6 g/L) by adding the peptone and/or yeast extract by an order of magnitude less with respect their amount in the conventional MB514, namely (three conditions carried out in triplicate): a) 10% of peptone (i.e. 0.5 g/L), b) 10% yeast extract (i.e. 0.1 g/L) and c) 10% of both (i.e. 0.5 g/L peptone + 0.1 g/L yeast extract);
- decrease of the salts concentration: a further experiment was conducted by employing a mineral medium with 50 % of the MB514 salts concentration (NaCl, Na_2SO_4, KCl, $NaHCO_3$, KBr, Na_2HPO_4, MgCl, CaCl, Fe-EDTA) except for the concentration of NH_4Cl, representing the main N source; according to the results of the previous experiment concerning the optimization of peptone and yeast extract, peptone was amended at 0.5 g/L while no yeast extract was used. Glucose was added at 6 g/L.

4.10 Test the growth of selected *Shewanella frigidamarina* in a mineral medium enriched in VFA-rich fermented cheese whey

In order to fed the selected strain with an actual VFA-rich fermentation broth, a cheese whey provided by Mambelli was processed under anaerobic acidogenic conditions. The obtained effluent contained 26 g/L of VFAs, mainly acetic (16%), propionic (24%) and butyric acid (60%). The *Shewanella frigidamarina* was grown in 500 mL shake flask (150 mL of optimised mineral medium), on a single condition carried out in triplicate. Overall, the flasks were spiked with the fermented cheese whey 6 times, so that about 0.3 g/L of VFAs were added by each spike, this leading to an overall addition of 2 g/L of VFAs in each flask. In agreement with results obtained by the experiments carried out for optimizing the composition of the growth medium (Section 4.9), a modified MB514 was employed by lowering the peptone content of an order of magnitude (0.5 g/L of peptone were added) while no yeast extract was used. The medium was amended with glucose (6 g/L) according to previous experiments where the growth of *Shewanella frigidamarina* was supported by this promptly available carbon and energy source.

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5 Results and Discussion

This section provides a structured overview of the activity's results, outlining the implementation phases and challenges encountered.

5.1 Screening of *Y. lipolytica* strains in minimal medium enriched with single commercial VFAs

The *Y. lipolytica* strains used in this work were previously tested and selected in several studies carried out by the Food Microbiology Group of DISTAL. In particular, strains RO25, Y3, RO3., PO2, PO1, RO2, PO10, RO21, PO19 and Y22A were chosen for their ability to grow well in cheese whey (Gottardi et al., 2023). Before growing the strains in complex mix of VFAs, strain-tolerance to acetic acid was evaluated. In fact, acetic acid is a toxic substrate for microorganisms [50] and therefore evaluation of strains capable to survive with and on it is fundamental. Strains were cultivated in minimal medium (YNB) supplemented with 0.2% acetic acid to determine their ability to adapt to its use as the sole carbon source. Turbidity was measured after three consecutive transfers through spectrophotometric analysis at 600 nm. Table 2 reports the absorbance values of the different *Y. lipolytica* strains.

Table 2. Absorbance (arbitrary units) measured at 600 nm after three consecutive transfers of the 10 strains of Y. lipolytica in YNB supplemented with 0.2% acetic acid

	Abs
PO17	0.20
RO2	0.29
RO21	0.32
RO25	0.01*
Y22A	0.01*
PO10	0.26
Y3	0.44
PO1	0.05*
PO19	0.14
PO2	0.12

This first test allowed the exclusion of strains PO1, RO25 and Y22A from further analyses, as they did not develop after three transfers. The remaining strains were then tested for growth at increasing concentrations of acetic acid according with the ranges proposed by Llamas et al., [4]. The absorbance variation over time reflected changes in cell biomass, enabling the definition of growth curves at room temperature for each strain and concentration of acetic acid. From Figure 4, a strain-specific growth behaviour was observed in response to the different concentrations of Strains RO2, PO2 and PO10 were the least affected, with stationary phase absorbance ranges of 0.2142–0.2549, 0.1798–0.2082 and 0.1809–0.2173, respectively. The highest variability was observed with strains Y3, RO21 and PO19, reaching absorbance values around 0.30 at the highest concentration of acetic acid. Conversely, strain RO3. behaved differently from all others: the highest absorbance (0.2186) was reached at the lowest concentrations of acetic acid, while at higher concentrations lower absorbances were recorded.

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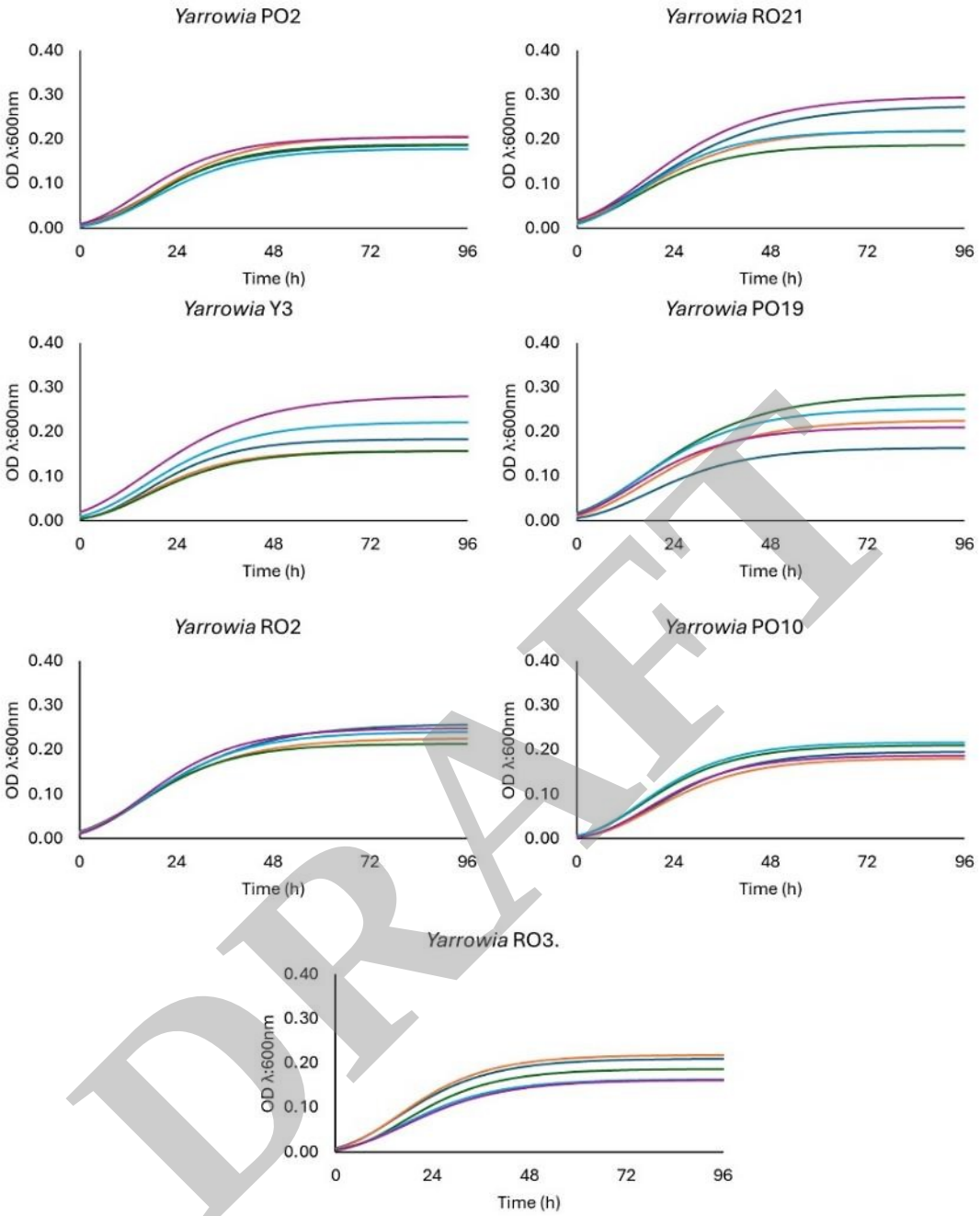

Figure 4. Growth curves of the different strains of Y. lipolytica PO2, RO21, Y3, PO19, RO2, PO10 and RO3. at different concentrations of acetic acid. The colour represents different concentrations in the range defined by Llamans et al., 2020.

The first two behaviors were rather unexpected since, in general, higher concentrations of fatty acids should correspond to lower microbial growth due to their inhibitory activity. Indeed, concentrations above 1% of short-chain fatty acids (SCFAs) exert inhibitory effects against both bacteria and yeasts [5]. However, in all the mentioned works the yeasts were not preadapted.

Based on these results, strains RO2, Y3 and RO3. were selected as representatives of the three different observed growth behaviors. These strains were further evaluated for their ability to grow in the presence of different concentrations of propionic and butyric acids. According to Gao et al. [5], increasing concentrations of these acids, when used individually, inhibit cell growth by chemically

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interfering with phosphate transport across the membrane, thereby increasing ATP consumption and hindering lipid accumulation.

Absorbance was monitored over time for acid concentrations. Fig. 5 shows the growth curves of strains RO3, Y3 and RO2 with both acids.

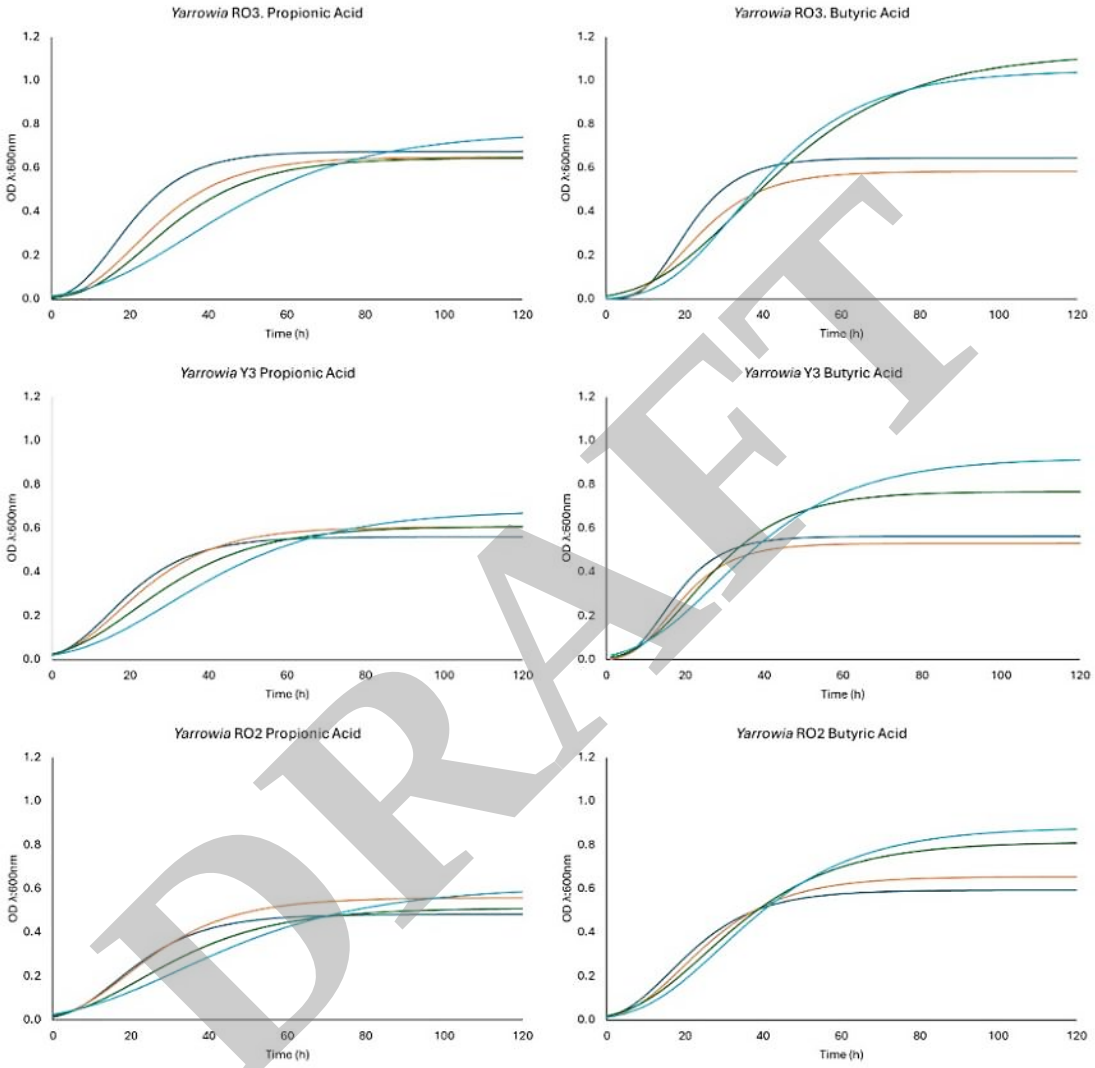

Figure 5. Growth curves of Y. lipolytica RO3., Y3 and RO2 at increasing concentrations of propionic acid (left) and butyric acid (right).

For strain RO3, growth in propionic acid showed relatively stable absorbance values in the range of concentration tested, with a marked increase at the highest. In butyric acid, RO3. reached very high absorbance at middle concentration (A = 1.12517) and the highest (A = 1.04425). For strain Y3, propionic acid led to moderate increases in A with middle-high concentrations. In butyric acid, strain Y3 achieved the highest absorbance values at the highest concentrations (from 0.76776 to 0.91899). For strain RO2, absorbance values in propionic acid ranged from 0.48 to 0.61 with the lower and highest concentration tested. In butyric acid, strain RO2 showed higher A values, up to 0.88 at the highest concentration tested.

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In general, butyric acid supported higher maximum absorbance (A) values compared to propionic acid. Among the strains, RO3. showed the strongest response in terms of maximum OD under butyric acid, whereas RO2 was generally less performant in propionic acid but reached higher values in butyric acid. Y3 appeared as an intermediate performer: less robust than RO3. under propionic acid, but capable of higher biomass accumulation in butyric acid than RO2. Its metabolic flexibility in the presence of butyric acid could make it interesting for applications involving mixed SCFA substrates. Unlike the results of Gao et al. [5], the strains tested in this study had been pre-adapted to acetic acid, which may explain why they did not show significant growth inhibition in the presence of increasing concentrations of butyric and propionic acids.

5.2 Growth and lipid production of 3 strains of *Y. lipolytica* in minimal medium enriched with a mix of VFAs

In real acetogenic fermented substrates, VFAs are not present as single compounds but rather as mixtures, due to the metabolic pathways involved in fermentation. For this reason, the growth and lipid production of the three strains of *Y. lipolytica* (Y3, RO3. and RO2) was evaluated in a minimal medium supplemented with a mixture of VFAs (acetic, propionic, butyric, valeric, isovaleric and caproic acids) according to [4].

Samples were incubated at low and room temperatures to evaluate biomass production, cell density, lipid accumulation, and lipid profile. Growth was first assessed by serial decimal dilution and plating on agar medium, followed by colony counting to determine cell density at different incubation times. The lower temperature was tested since Patrignani et al. reported the presence of PUFA in pork fat after 21 days of storage at 15 °C with *Y. lipolytica* [51]. It is also known that microorganisms increase the percentage of unsaturated fatty acids when are grown at low temperature [52] (Russell, 2008).

Spectrophotometric analysis at 600 nm showed that at RT, absorbance increased until day 6, decreased slightly until day 10, and rose again by day 13 (Fig. 6). At LT, absorbance decreased until day 10, then increased sharply until day 20, reaching the highest values in strain RO2 (1.062), followed by RO3. (0.822) and Y3 (0.734) (Fig. 7.8). These oscillations may be related to the polymorphic nature of *Y. lipolytica*, which can switch between unicellular, hyphal and pseudo-hyphal morphologies, potentially affecting spectrophotometric measurements.

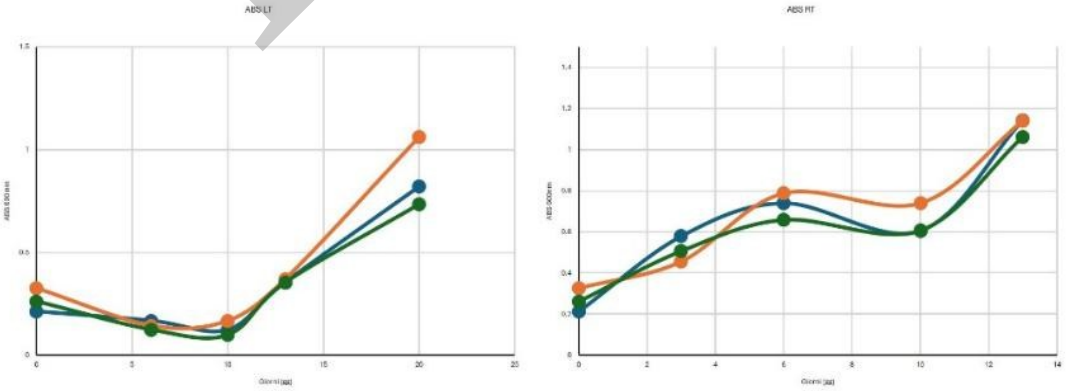

Figure 6. Absorbance at 600 nm of the cultures of Y. lipolytica (orange RO3.; light orange RO2; gray Y3)) grown in YNB + a mix of VFAs for up to 13 and 21 days at room temperature and low temperature, respectively.

Biomass dry weight (Fig. 7) showed similar values among strains at RT (≈700 mg/100 mL), while at LT strain Y3 produced significantly more biomass (817.86 mg/100 mL) compared to RO3. (741 mg/100 mL). These values were higher than those reported by Gao et al. [5], where biomass yields ranged from 2–3 g/L. Similarly, Llamas et al. [4] observed biomass production 2.4 times higher in real anaerobic digestate compared to synthetic medium. Thus, the results obtained in this work suggest potentially higher yields when applied to complex substrates such as food by-products.

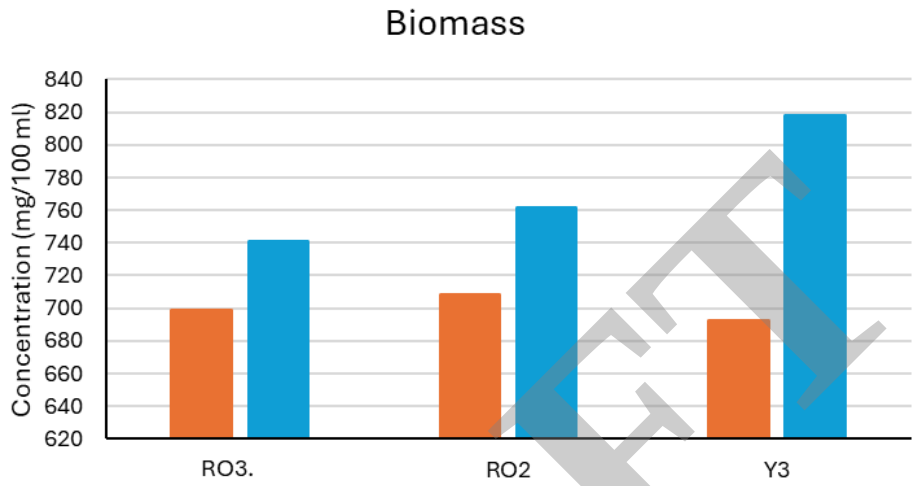

Figure 7. Biomass dry weight of the different Y. lipolytica strains grown in YNB with a mix of VFAs. Orange: Room Temperature 13 days, blue: Low Temperature 20 days

Fig. 8 shows lipid production (mg/100 mL broth). At RT, strain Y3 produced the highest lipid amount (79.8 mg/100 mL), while RO3. showed the lowest (11.2 mg/100 mL). At LT, lipid content dropped drastically: RO2 (8.0 mg/100 mL), RO3. (6.4 mg/100 mL), Y3 (4.5 mg/100 mL).

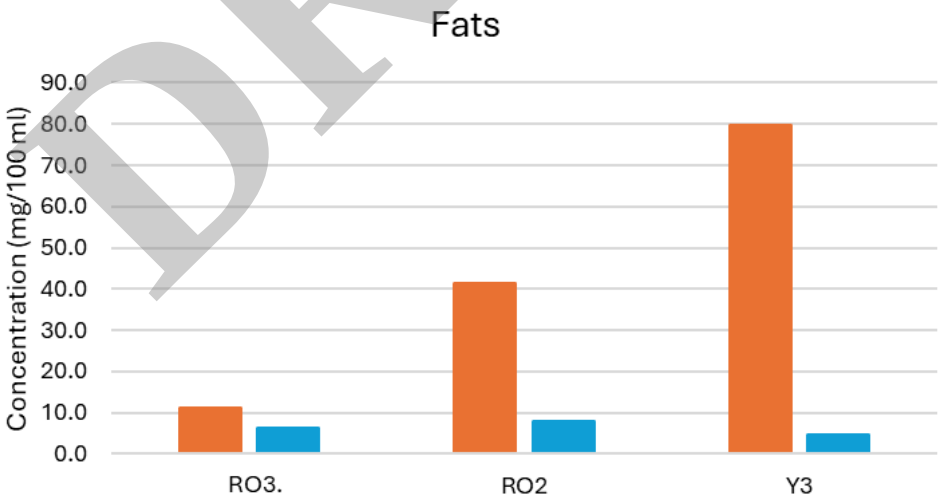

Figure 8. Lipid content from the biomass of Y. lipolytica grown in YNB + mix VFAs at RT or LT for 13 or 20 days, respectively. Orange: Room Temperature 13 days, blue: Low Temperature 20 days

When normalized to biomass (Fig. 9), the lipid-to-biomass ratio was lower than in Gao et al. [5]. At RT, strain Y3 reached 11.53%, while RO2 and RO3. showed 5.86% and 1.60%, respectively. At LT, lipid

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content remained below 1%, suggesting that fatty acids were primarily used to sustain growth rather than stored as reserves.

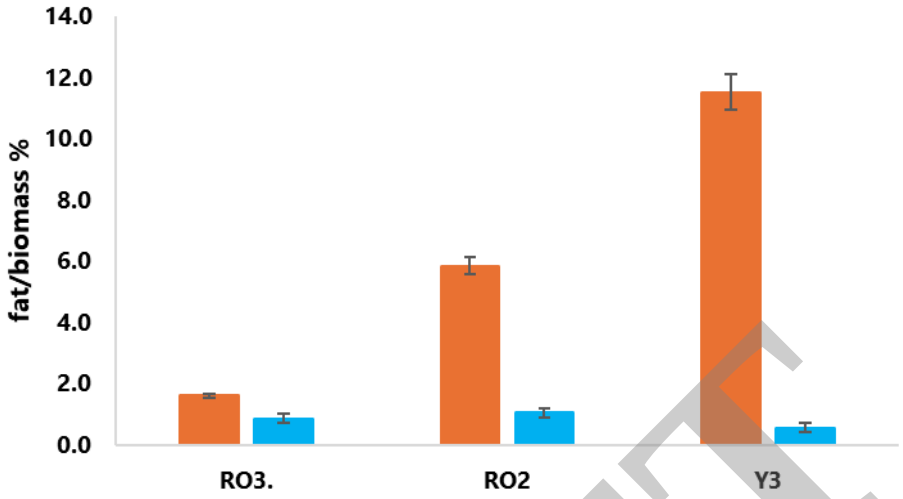

Figure 9. lipid-to-biomass ratio of Y. lipolytica grown in YNB + mix VFAs at RTt or LT for 13 or 20 days, respectively. Orange: Room Temperature 13 days, blue: Low Temperature 20 days

As one of the main goal of ONE-EARTH project is to produce (P)UFAs by microrganisms starting from an acidogenic fermented whey. Therefore, Fatty acids composition is also very important. Fatty acid profiling revealed that at RT, the most abundant fatty acids were C18:1 (20.3–23.4%), C18:2 (16.1–20.6%), C9:0 (6.6–15.8%), C10:0 (9.7–15.2%) and C16:0 (6.5–11.2%) (Fig. 7.12). Compared to Gao et al. [5], where C18:1 (30.9%), C18:0 (18.3%) and C16:0 (18.9%) dominated, the strains in this study showed higher unsaturation levels, particularly RO3. and RO2 (55.5% and 53.1%) compared to Y3 (47.6%).

As expected, at LT, fatty acid profiles shifted towards an increased unsaturation (0.87–0.98 vs. 0.69–0.82 at RT). RO3. exhibited the highest levels (UFA: 66.6%; PUFA: 29%), followed by RO2 and Y3. This increase was mainly due to higher C18:2 (21.7–26.6%), reduced C18:1 (18.4–21.7%) and increased C16:1 (10.9–13.1%) (Figs. 10). Long-chain fatty acids (C20:3, C22:2) were detected at both temperatures in the range of 0.2–0.6%.

According to these data, *Y. lipolytica* Y3 was selected to be tested in more real environments.

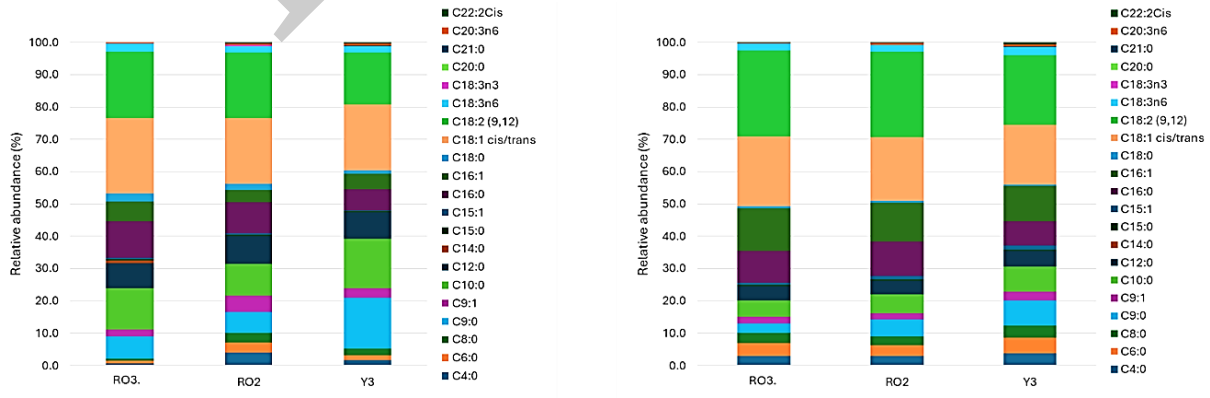

Figure 10. Relative abundance of the fatty acids produced by the different strains of Y. lipolytica at the end of the incubation period at 13 and 21 days at RT (left) and LT (right).

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5.3 Evaluation of *Y. lipolytica* growth and lipid accumulation in acidogenic fermented whey

The trial was repeated using *Y. lipolytica* Y3 incubated for 3 and 13 days at RT in static or stirring conditions using cheese whey provided by UNIVPM as substrate. The cheese whey, obtained from acidogenic fermentation of squacquerone for 5 days had the following composition (Tab. 3).

Table 3. VFA composition of the cheese whey provided by UNIPM

	Lactose (g/L)	Galactose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)
Squac-5d	0.00	0.14	5.88	0.71	0.42

As shown in Fig. 11, constant aeration did not improve lipid yield. On the contrary, stirring conditions limited lipid accumulation (maximum 13% CDW after 13 days), while negatively affecting product quality due to the development of undesirable smell and dark coloration. Conversely, static incubation strongly promoted the accumulation of single-cell oils (SCOs) with respect to the dry biomass. After 13 days, lipid content reached 42% of CDW, representing a more than three-fold increase compared to aerated conditions.

Figure 11. Lipid-to-biomass ratio of Y. lipolytica Y3 grown in acidogenic fermented squacquerone whey in stirring or static conditions for 3 and 13 days at RT.

The fatty acid composition of the accumulated lipids (Fig. 12) showed a strong prevalence of C18 fatty acids. In particular, after 13 days under static conditions, Y. lipolytica Y3 produced lipids enriched in long-chain unsaturated fatty acids, mainly C18:1, C18:2 and C18:3, which together accounted for more than 50% of the total fatty acids. No fatty acids with chain length longer than C18 were detected.

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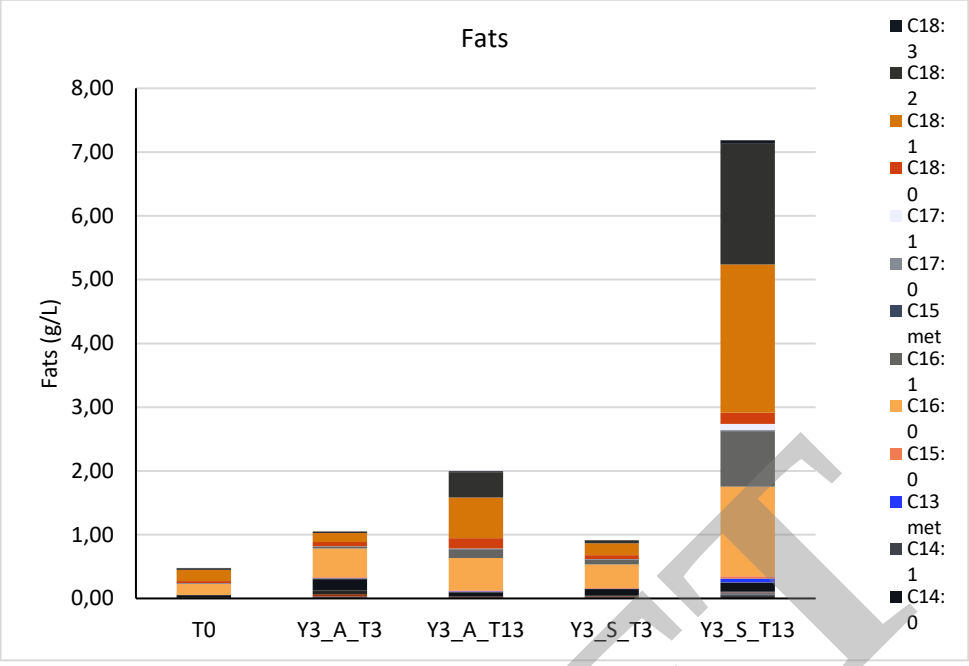

Figure 12. Fat content and fatty acid composition of the SCO produced by Y. lipolytica in static (S) or stirring (A) condition after 3 and 13 days of incubation at RT.

The analysis of volatile fatty acids (VFAs) in cheese whey during incubation with *Y. lipolytica* showed that their overall consumption was more pronounced after 13 days compared to 3 days (Tab. 4). Nevertheless, in static conditions an unexpected increase in butyric acid was observed, suggesting that additional microbial activities may have occurred alongside *Y. lipolytica* metabolism. This behaviour is not surprising given that the system was not sterile, and thus other microorganisms likely contributed to the VFA dynamics.

Table 4. Composition of the cheese whey at the end of incubation with Y. lipolytica Y3

Sample	Acet.	Prop.	But.	Iso-but.	Val.	Iso-val.	Cap.	Total
S.Y3_T0	1.78	0.42	0.16	0.00	0.00	0.05	0.06	2.47
A.Y3_T3	0.81	0.03	1.02	0.00	0.00	0.05	0.05	1.96
S.Y3_T3	0.85	1.17	0.04	0.00	0.00	0.05	0.06	2.17
S.Y3_T13	0.29	0.78	0.49	0.00	0.00	0.00	0.01	1.57

5.4 Growth and fat production of *Y. lipolytica* Y3 in squacquerone enriched in VFAs produced with and without *Propionibacterium* provided by UNIVPM or with Kefir grains by DISTAL-UNIBO

Since UNIPM performed different strategies of fermentation (natural fermentation, guided fermentation with *Propionibacterium freudenreichii*), these were evaluated as substrates for the growth and lipid production of *Y. lipolytica* Y3. Moreover, UNIBO-DISTAL produced a Kefir-fermented whey rich in acetic acid. The initial VFA composition of the substrates is reported in Table 5.

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Table 5. Main VFAs composition expressed as ppm equivalent to internal standard of the different substrates provided by UNIVPM at the beginning (T0) and end of fermentation (T3) in agitation (A) or not.

	Natural-fermented whey			PP-fermented whey			Kefir-fermented whey		
	T0	T3	AT3	T0	T3	AT3	T0	T3	AT3
acetic acid	2.05	0.03	0.04	1.86	0.31	0.04	0.34	0.35	0.04
propionic acid	2.63	0.06	bdl	7.99	1.21	bdl	Bdl	bdl	Bdl
butanoic acid	98.14	0.68	0.08	85.43	14.36	0.25	bdl	0.32	bdl

From an industrial perspective, it was recommended to carry out the fermentation with *Y. lipolytica* in the shortest time possible; therefore, the experiment was repeated with a duration of only three days. Furthermore, the samples were filtered down to 0.2 µm by UNIPM to ensure microbiological stability and to allow a clearer evaluation of the yeast's impact on the final product.

Among the tested substrates, naturally-fermented whey proved to be the most favorable for yeast development, allowing *Y. lipolytica* Y3 to reach up to 8 log CFU/mL within three days of incubation, independently of whether the system was kept under static or stirred conditions (Fig. 13). Kefir- and PP-fermented whey, although able to sustain yeast growth, generally resulted in lower cell densities, highlighting the impact of substrate composition and the presence of organic acids on yeast metabolism.

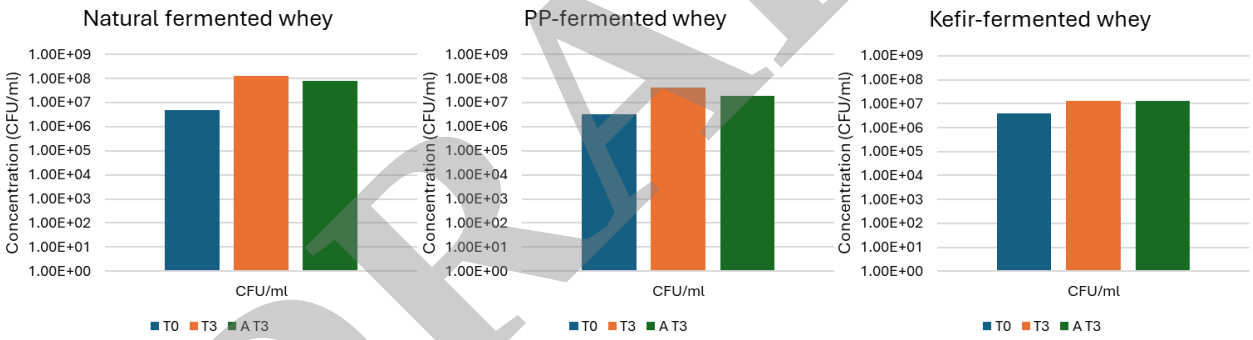

Figure 13. Cell count of Y. lipolytica in the different substrates

The analysis of lipid accumulation revealed distinct patterns depending on the incubation conditions (Fig. 14). Under static conditions, the yeast produced a higher amount of lipids when incubated in natural-fermented whey (almost 160 mg/L of whey). In contrast, PP-fermented whey and Kefir-fermented whey produced a higher concentration of fats in agitation (around 60 mg/L compared 40 mg/L or lower).

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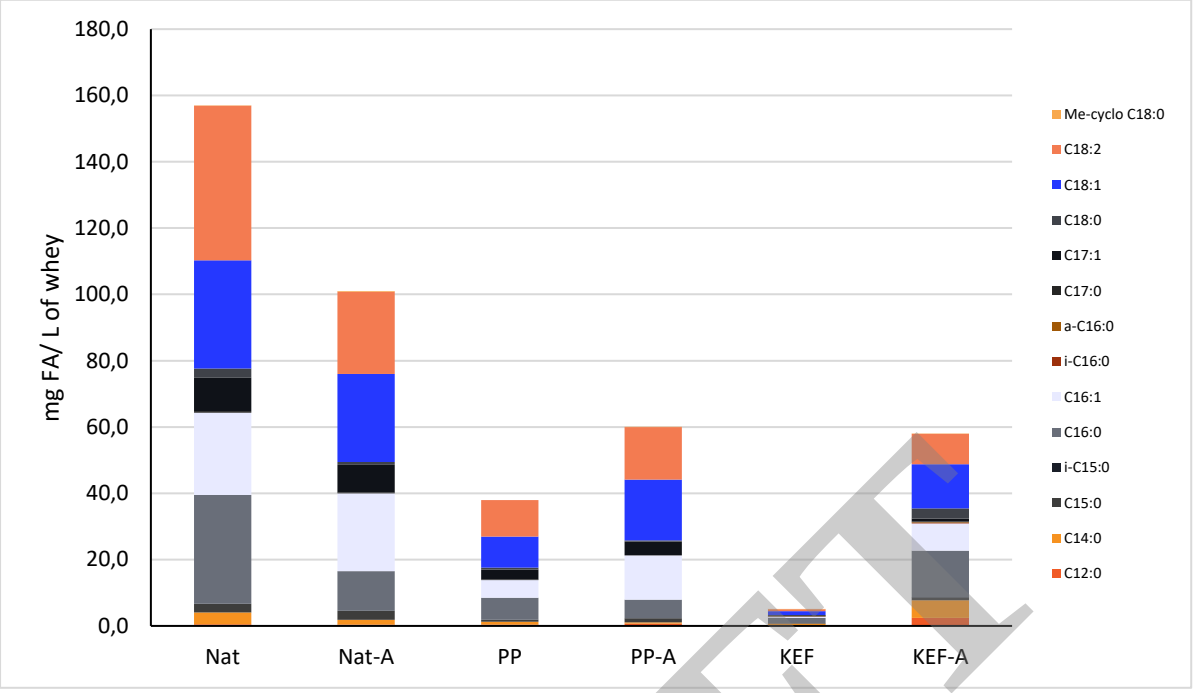

Figure 14. Fatty acid content and composition after 3 days of incubation of Y. lipolytica Y3 under shaking (A) or static conditions in the three different substrates.

Looking at the composition, stirring favoured the accumulation of a more unsaturated fatty acid profile, with up to 85% of the total fatty acids being unsaturated (Fig. 15). The main components were C18:2, C18:1, C16:1 and C17:1, which together represented the majority of the lipid fraction (Fig. 14). These findings confirm the strong metabolic flexibility of *Y. lipolytica*, which can adjust its lipid composition depending on environmental conditions. From a composition point of view, no differences were observed in saturated/unsaturated FA when *Y. lipolytica* Y3 was grown in Natural-fermented whey or PP-fermented whey, while differences were observed in kefir-fermented whey. In this case, the percentage of saturation was higher.

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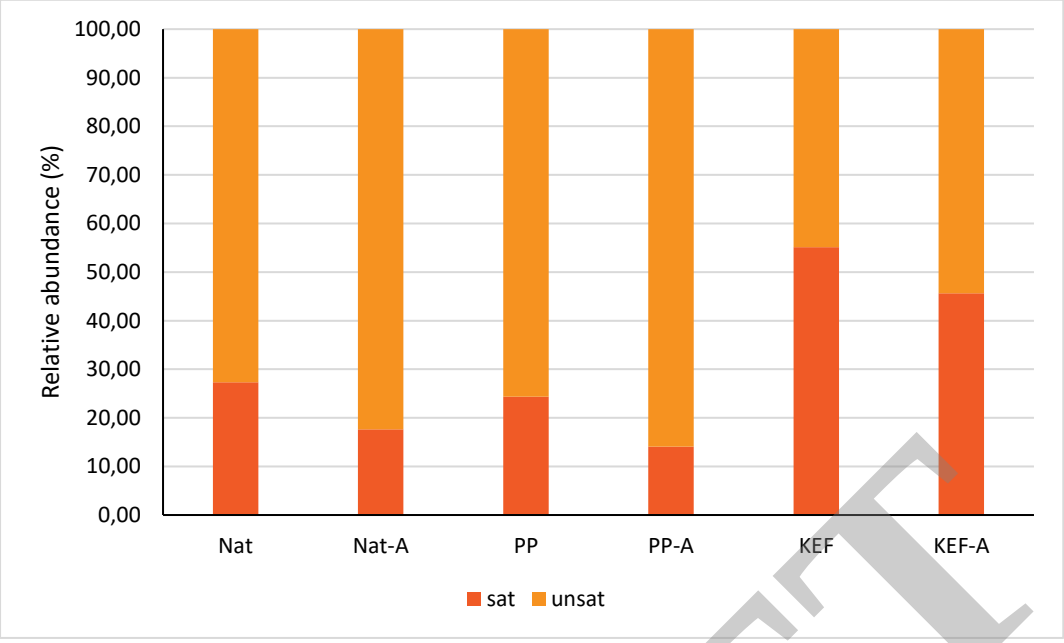

Figure 15. Relative abundance (%) of saturated and unsaturated fatty acids in the different samples.

Biomass production was particularly high in kefir-fermented whey under aerated conditions, where dry weight values exceeded 13 g/L. In the other samples, values were in the order of 5.7 g/L in natural-fermented whey and 3.7 g/L in PP-fermented whey. The higher value subserved in Kefir-fermented whey may be overestimated due to the characteristics of the kefir-fermented whey which was not filtered compared to the other substrates.

Looking at the percentage of fat with respect to the CDW, the higher yield was measured for natural-fermented whey in static condition with a 2.7 % (fat/CDW). This is not a high value compared to what *Y. lipolytica* can do but it is in line with the previous results obtained within the time of incubation considered. Longer incubation time may enhance the accumulation.

Looking at the evolution of VFAs, it can be observed that under shaking conditions (A) almost all acids dropped below the detection limit, whereas under static conditions small residual amounts were still detectable. This suggests that aeration enhanced the metabolic activity of *Y. lipolytica* Y3, leading to a more efficient utilization of volatile fatty acids compared to static incubation but they were used more for cell growth than lipid accumulation.

Overall, the study demonstrates that *Y. lipolytica* Y3 can efficiently convert different types of fermented whey into microbial oils enriched in unsaturated fatty acids. Natural whey emerges as the most promising substrate, providing a good compromise between growth performance, lipid content and fatty acid quality. Although PUFAs were not produced, these results confirm the biotechnological potential of integrating dairy by-products with oleaginous yeasts to obtain sustainable microbial oils rich in nutritionally valuable fatty acids, which can be exploited for food, feed and industrial applications.

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5.5 Test the growth and PUFAs production of *Y. lipolytica* RO3 strain using a modified YPD medium supplemented with glucose or glycerol and pulses of commercial VFAs solutions

After the yeast strains screening tests performed by UNIBO, RO3 and Y3 strains were selected to be sent to Biotrend for further development experiments. RO3 was the first yeast strain to be tested using Biotrend's fermentation conditions. Since, the FA production capacity of *Y. lipolytica* strains is enhanced by the lower nitrogen content of the medium, a modified YPD medium with lower nitrogen concentration (yeast extract and peptone) was used. To enhance the biomass production in this growth study, the YPD medium was supplemented with glucose (20 or 40 g/L) or glycerol (20 g/L), the two main carbon sources most used in *Y. lipolytica* fermentations (see Section 4.5). During the growth study, the biomass production was monitored by OD_{600nm} analysis and, when a slowdown on yeast growth was observed, a 2 g/L pulse of sugar or commercial VFA was added to evaluate the impact of this pulse on yeast growth and FA production (more procedure details are present on Section 4.5). The VFAs used in this study were acetic, butyric and propionic acids, the major components of the fermented whey that is being produced by UNIVPM.

On Figure 16 are presented the yeast growth results and sugars/acids concentrations in the medium during this experiment. Independently of the medium conditions tested, glycerol was present in all shake flasks because the yeast cryopreservation was performed using glycerol as cryopreservation agent. 6 and Figure 17 shows the final CDW and FA production results (FA content in biomass and FA profile).

This growth study showed that yeast strain RO3 was able to growth in all medium conditions tested. Glycerol was the yeast preferred carbon source, being faster consumed and causing the slowdown on yeast growth observed after 48 h of EFT (Figure 16). Due to the presence of glycerol and the lower nitrogen content in all media tested, glucose consumption rate was slower, especially when higher glucose concentration was used (40 g/L, test 6).

The sugars/VFAs pulses added after 48 h EFT enabled to confirm that there is no significant impact on yeast growth after glucose or glycerol pulses addition. After VFAs pulses addition, a slowdown on yeast growth was observed for each of the three acids tested. However, culture growth restarted some hours later (glucose was still present in all media) and VFAs were consumed at different rates (Figure 16). Faster VFAs consumption was observed in the medium with acetic acid and two pulses were added until the end of the experiment, since the first pulse was completely consumed. RO3 yeast strain took more time to metabolize butyric acid, and its consumption was only observed in the last 24 h of the growth study. Regarding the use of propionic acid, this acid was not consumed by this yeast strain using the fermentation conditions used in this growth study.

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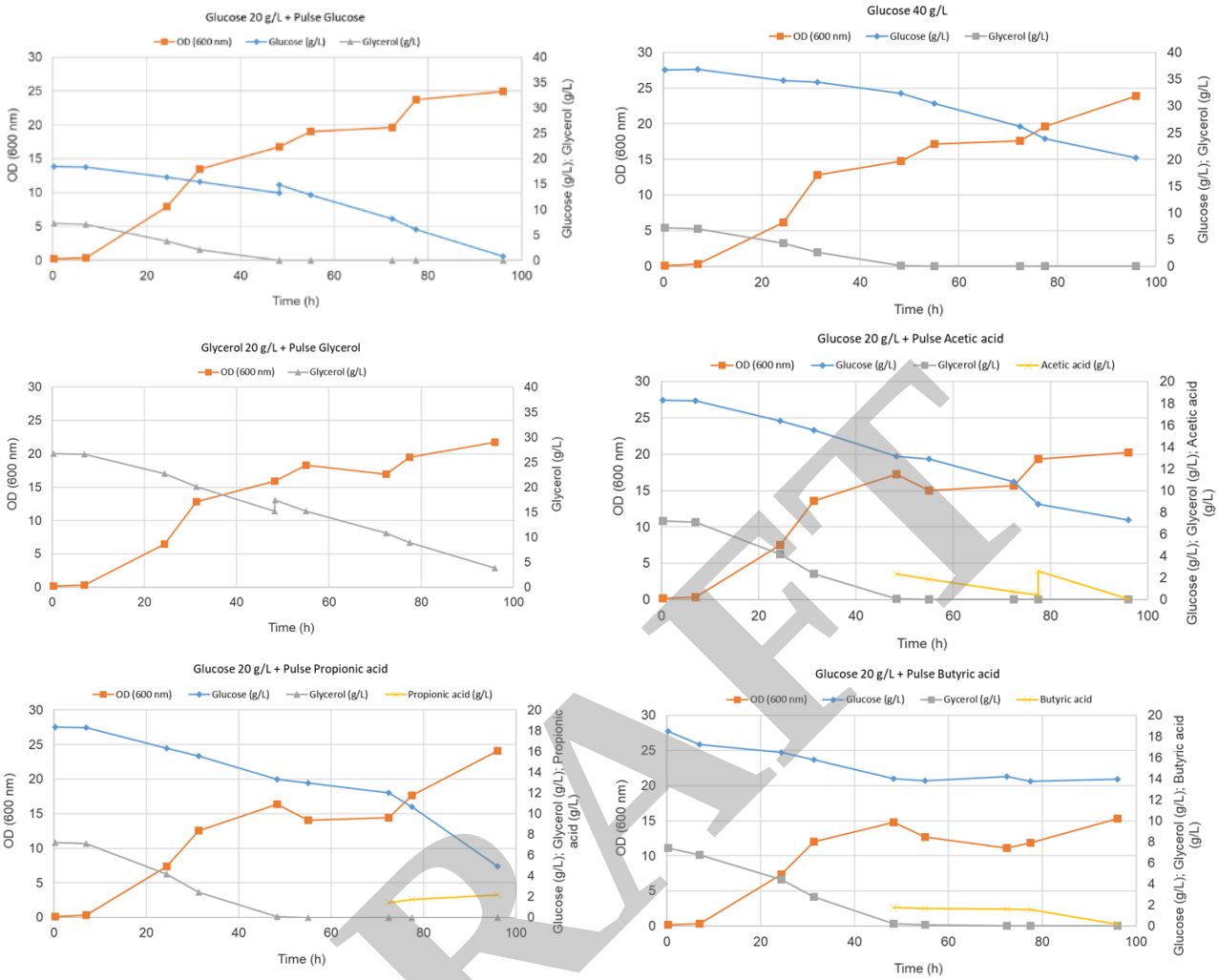

Figure 16 – RO3 yeast growth and sugars/acids concentration results of the growth study performed using modified YPD and different carbon source conditions.

Table 6 – Final CDW results obtained in RO3 yeast growth study.

Test ID	Carbon source initial medium	Carbon source pulse	CDW (g/L)
1	20 g/L glucose	2 g/L glucose	11.85
2	20 g/L glucose	2 g/L acetic acid	9.30
3	20 g/L glucose	2 g/L propionic acid	10.80
4	20 g/L glucose	2 g/L butyric acid	5.85
5	20 g/L glycerol	2 g/L glycerol	10.45
6	40 g/L glucose	-	11.30

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The use of VFAs in RO3 yeast fermentations had a negative impact on the final biomass produced (Table 6). While in glucose or glycerol media, 10.45-11.85 g/L of dry biomass was produced, only 5.85-10.8 g/L of dry biomass was produced when VFAs were added to the fermentation medium. The lowest biomass production was obtained using butyric acid.

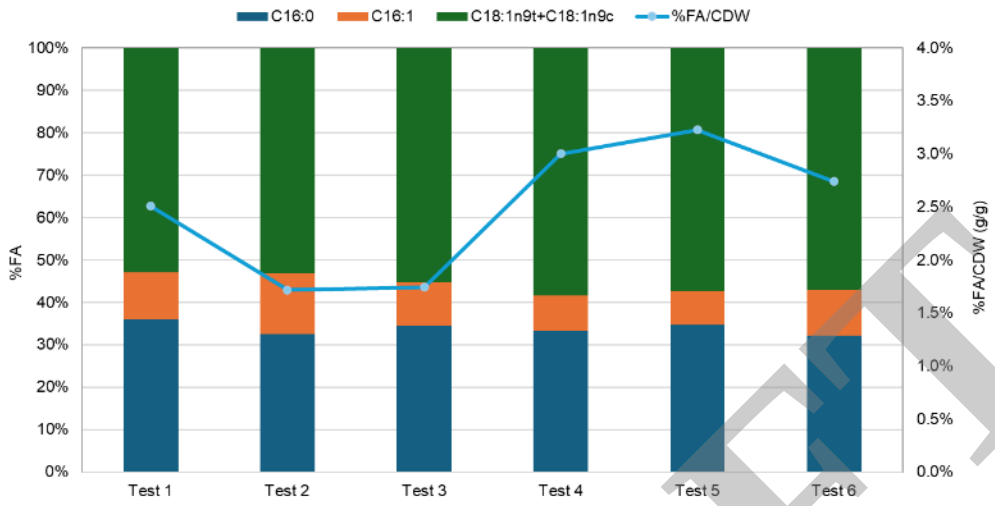

Figure 17 – FA content in biomass and FA profile results of the final RO3 yeast biomass produced.

Despite the negative impact of VFAs on yeast biomass production, no significant differences were found on FA production and lipids composition (Figure 17). 1.57–3.35% of FA content in biomass was attained in all conditions tested, with the highest values being obtained in butyric acid and glycerol tests. The FAs content observed in this study was similar with the results obtained by DISTAL-UNIBO for RO3 strain using different cultivation conditions: 1–2% (see Section 5.2). The FA profile was also similar between the medium conditions tested in this growth study: C16:0 (25.3-41.1%), C16:1 (7.7-17.7%) and C18:1 (50.2-66.7%).

The growth test of RO3 *Y. lipolytica* strain using different fermentation conditions used in the previous experiments performed by UNIBO, namely the medium composition, demonstrates the high process robustness and versatility of this yeast strain for biomass and FA production. Despite the low FA content in biomass obtained, this yeast strain was able to metabolise VFAs, especially acetic and butyric acids, independently of the fermentation medium used.

This shake flasks experiment represented the beginning of the fermentation process development to be held by Biotrend to prepare the process scale-up activities: task 2.2.2 and TRL 5 of One-Earth project. More development studies will be done using also the Y3 *Y. lipolytica* strain, which was meanwhile selected from UNIBO's work, as the most promising yeast strain for further process development.

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5.6 Selection of bacteria potentially capable of producing target PUFAs.

- 5.6.1 among organisms that were mentioned as PUFA producers throughout scientific papers, algae and protists are the mainly represented groups. Among bacteria, *Moritella marina* species were studied the most, being used as positive control for docosahexaenoic acid (DHA) production, too. Beside *Moritella* sp., *Shewanella* sp., and in particular *S. pacifica* and *S. frigidimarina* strains, are indicated as feasible EPA producers. As the result of the literature review, the three mentioned bacteria were acquired by UNIBO and UNIPR as commercial alternatives to bacteria isolated and available by the two partners.
- 5.6.2. Three marine bacteria belonging to UNIBO that were selected according to previous findings (G16.20, P11.20 and P16.20 [15]) were tested according to the procedure described in Section 4.6.2. No target PUFA productions were observed. As an example, a series of chromatographic profiles related to G16.20 are reported in Fig. 18, where the target PUFA elution zone is highlighted.

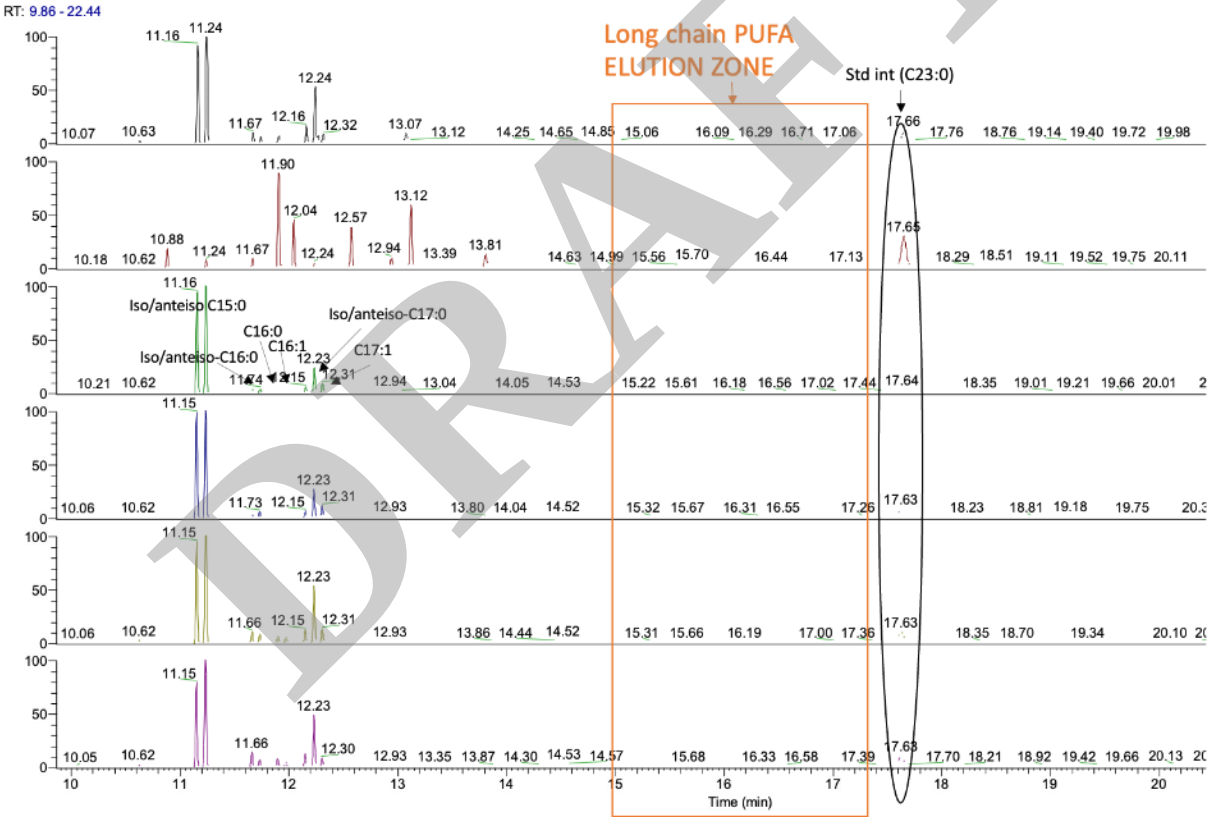

Figure 18. Fatty acids chromatographic profiles for the analyses related to strain G16.20

- 5.6.3 UNIPR performed an *in silico* analysis on specific species of deep-sea microorganisms, which are reported in the literature for their potential ability to produce PUFAs, to assess the presence of the proteins responsible for PUFAs production. Bioinformatic analysis was carried out to evaluate the presence of the proteins responsible for PUFAs production in the proteome of *Shewanella pacifica*, *Shewanella frigidimarina* and *Moritella marina* species.

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Table 7. Bioinformatic analysis showing query cover percentage and identity percentage of protein belonging to S. gelidimarina and S. pacifica found through alignment with pfaA-E proteins belonging to the cognate microorganisms S. sp. And S. pneumatophori.

Protein	Reference protein	*Shewanella frigididimarina*			*Shewanella pacifica (japonica)*		
		Protein	Query cover	% Identity	Protein	Query cover	% Identity
PfaA	eicosapentaenoate synthase subunit PfaA [Shewanella pneumatophori] WP_248949626.1	type I polyketide synthase WP_248987560.1	100%	78.87%	type I polyketide synthase WP_119968764.1	100%	65.73%
PfaB	PfaB [Shewanella sp. BR-2] ACI12949.1	PfaB family protein WP_346347756.1	92%	44.17%	PfaB family protein WP_282167673.1	90%	46.97%
PfaC	PfaC [Shewanella sp. BR-2] ACI12950.1	eicosapentaenoate synthase subunit PfaC WP_248987561.1	99%	67.38%	beta-ketoacyl synthase N-terminal-like domain-containing protein WP_119968766.1	99%	69.47%
PfaD	PfaD [Shewanella sp. BR-2] ACI12951.1	eicosapentaenoate synthase subunit PfaD WP_248987562.1	98%	82.90%	eicosapentaenoate synthase subunit PfaD WP_282167671.1	98%	84.97%
PfaE	eicosapentaenoate biosynthesis 4'-phosphopantetheinyl transferase PfaE [Shewanella pneumatophori] WP_125489406.1	4'-phosphopantetheinyl transferase superfamily protein MCL1057673.1	99%	52.52%	4'-phosphopantetheinyl transferase family protein WP_156003184.1	91%	50.20%

Table 7 shows the results of the alignment between potential pfaA-E proteins, responsible for PUFAs production (Moi et al., 2018), belonging to our microorganisms of interest (*S. pacifica* and *S. frigidimarina*) with the same known proteins found in cognate microorganisms (*Shewanella* sp. and *S. pneumatophori*). Query cover percentage and identity percentage values were reported, resulting in a high coverage of the aligned sequences for all the proteins of both *S. pacifica* and *S. frigidimarina* (between 90% and 100%). Regarding the identity percentage, the values range from 44% to 86%, indicating a partial identity; however, the identical portions are usually referred to the active sites, thus indicating a potential similar role between the two aligned proteins.

The same research was also performed for *M. marina* also (Table 8). In this case, pfaB-D-E were directly found through NCBI research, for this reason just the ID number of the protein stored in the databank is reported; while a high identity percentage (86/87%) was detected between known pfaA and pfaC belonging to Moritella viscosa, used as reference, and proteins found in Moritella marina, thus probably having the same function.

Accordingly, based on the in-silico analysis, all the microorganisms of interest could be potentially capable of producing PUFAs, since they could possess the needed proteins in their proteome.

- 5.6.4 Between the tested microorganisms (Section 4.6.4), 3 of them (belonging to *Agrococcus* and *Planomicrobium* genera) were positive to the test (Fig. 19), meaning that were able to grow regardless of all the concentrations of H2O2 employed. These microorganisms were further tested through GC-MS to evaluate the presence of PUFAs, but unfortunately the latest were not observed.

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Table 8. Bioinformatic analysis showing query cover percentage and identity percentage of protein belonging to Moritella marina found through alignment with pfaA-E proteins belonging to the cognate microorganism Moritella viscosa.

Protein	Reference protein	*Moritella marina*		
		Protein	Query cover	% Identity
PfaA	putative polyunsaturated fatty acid synthase [Moritella viscosa] CED60045.1	SDR family NAD(P)-dependent oxidoreductase [Moritella marina] WP_392339844.1	99%	86.85%
PfaB		WP_392339845.1 WP_019440099.1		
PfaC	putative polyunsaturated fatty acid synthase [Moritella viscosa] CED60047.1	beta-ketoacyl synthase N-terminal-like domain-containing protein [Moritella marina] WP_392339846.1	100%	87.93%
PfaD		WP_392339847.1		
PfaE		BAF02836.1		

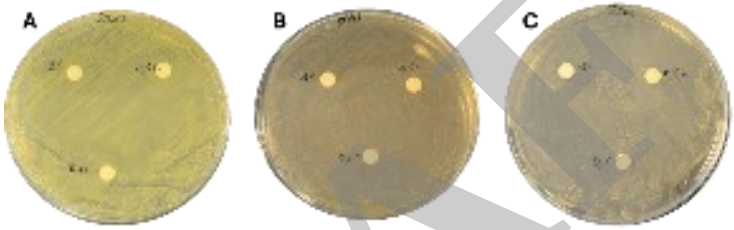

Figure 19. Positive strains to the H2O2 test.

The resistance to the oxidative action of H2O2 could be ascribed to the presence of squalene, which was detected during GC-MS analysis.

5.7 Screening the growth of selected bacteria to verify their ability of producing target PUFAs

The three bacterial strains selected according to the literature review and the *in-silico* analyses (*M. marina* DSM 104096, *S. frigidimarina* DSM 12253 and *S. pacifica* DSM 15445, as mentioned in the previous Section 5.6.3), were tested in media amended with glucose by both UNIBO and Biotrend (Section 4.7). Results obtained by UNIBO are reported in Table 8 in terms of relative lipid compositions. As expected, *Shewanella* spp. were able to accumulate EPA among target PUFAs (4.47 and 3.34%, w/w, for *S. pacifica* and *S. frigidimarina*, respectively), while the *Moritella* sp. produced DHA (5.64%). Unfortunately, none of them accumulated significant amounts of both PUFAs, although some EPA acid was observed in the *Moritella* biomass. The highest overall lipid content was observed in the *S. pacifica* (about 9%); however, these amounts refer to non-optimized cultivation conditions.

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Table 9. Fatty acids concentrations detected in the grown biomasses of marine bacteria, which were employed in experiment for assessing their ability of producing target PUFAs

	S. pacifica	*M. marina*	*S. frigidimarina*
FAME peak	**rel %**	**rel %**	**rel %**
C10:0	0,00	0,00	0,00
C12:0 (lauric)	3,53	1,12	3,12
C13:0 iso	6,06	0,00	7,43
C13:0	1,06	0,00	1,25
cyc-FA	0,00	1,70	0,00
C14:0 (iso)	0,82	0,00	1,17
C14:0 (myristic)	4,92	14,88	5,86
C14:1 isomer 1	0,44	0,82	1,00
C14:1 isomer 2	0,00	8,71	0,00
C14:0 ox (Methyl 3-methoxytetradecanoic)	1,17	0,00	0,31
C15:0 anteiso	14,48	0,84	9,34
C15:0 iso	3,22	1,41	0,89
C15:0	3,17	0,91	4,89
C15:1 isomer 1	0,38	0,44	1,17
C15:1 isomer 2	0,42	0,00	1,12
C16:0 iso	0,00	0,00	0,00
C16:0 (palmitic)	13,15	15,26	12,72
C16:1 sum of isomers	14,23	33,21	20,65
C17:0 anteiso	0,00	0,50	0,00
C17:0 iso	2,32	0,00	1,12
C17:0	2,15	0,00	2,54
C17:1 isomer 1	7,87	0,00	7,66
C17:1 isomer 2	0,97	0,00	1,17
C18:0 (stearic)	2,29	1,32	1,90
C18:1 isomer 1	3,91	1,59	4,29
cis-9 C18:1 (oleic)	8,76	8,30	6,35
C18:2	0,00	0,00	0,00
C19:1/cyp-C19:0	0,00	0,00	0,00
cyp-C19:0	0,00	0,00	0,00
ω3-C18:4	0,21	0,00	0,59
ω6-C20:4	0,00	0,61	0,00
C20:5 (EPA)	4,47	0,72	3,44
C22:6 (DHA)	0,00	5,64	0,00
lipid yelds (% biomass)	*9,00*	*7,40*	*4,00*

In parallel, the development of PUFAs production process mediated by bacteria started in Biotrend by screening the same strains experiment in shake flasks scale. Only *S. frigidimarina* was able to growth on the fermentation conditions used. The bacteria growth and glucose concentration results are presented in **Errore. L'origine riferimento non è stata trovata.**20.

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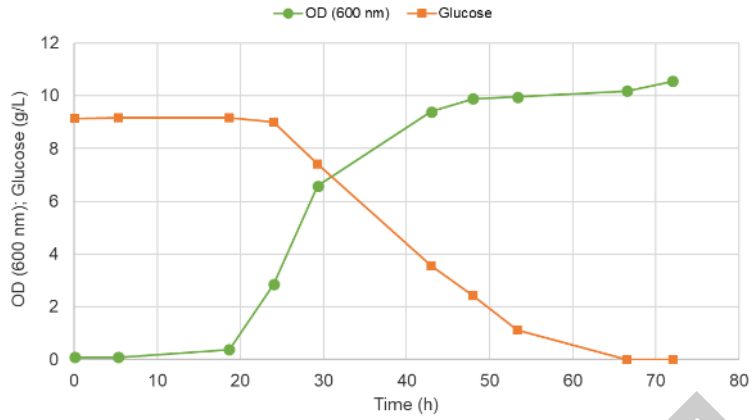

Figure 20 – S. frigidimarina growth profile and glucose concentration obtained in the screening shake flask experiment.

After 72 h of incubation, no growth was observed for *M. marina* and *S. pacifica* strains.

During the *S. frigidimarina* small scale fermentations performed by UNIBO, marine broth 2216 and modified versions of this medium were used. Those media have a different nutrients composition of the rich medium used by Biotrend on previous screening experiment (Section 4.7). To evaluate the impact of using different fermentation media on bacteria biomass and PUFAs production, a new shake flask experiment was performed. The main results of this activity is presented on **Errore. L'origine r iferimento non è stata trovata.**21.

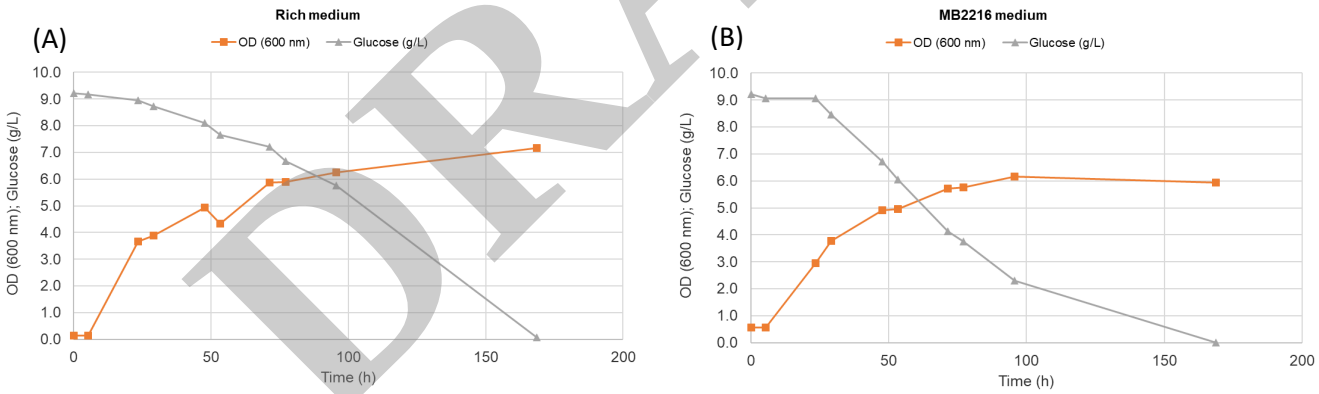

Figure 21. Biomass growth profile and glucose concentration observed in S. frigidimarina growth study using different culture media: (A) rich medium; (B) marine broth.

The bacteria showed similar growth profiles for both media used. However, more biomass was produced using the rich medium, 3.55 g/L. 2.85 g/L was the final biomass production obtained with marine broth, but the biomass produced contained higher salts content due to the medium precipitation observed after medium preparation and sterilization. The glucose consumption profile was also similar between the two media tested.

The use of two different media on *S. frigidimarina* fermentations could trigger different metabolism routes on bacteria, resulting in the production of different PUFAs or byproducts. For example, it is

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known that *S. frigidimarina* could produce red pigments under specific fermentation conditions [53]. On **Errore. L'origine riferimento non è stata trovata.**22 is shown the different colours observed on t he culture broth, at the end of the growth study. The differences found on bacteria biomass colour could be a result of the possible metabolic changes between the two tested media.

No PUFAs production and lipids profile results can be presented in this deliverable, since these analysis are being performed at the moment of the submission of the deliverable.

(A) (B)

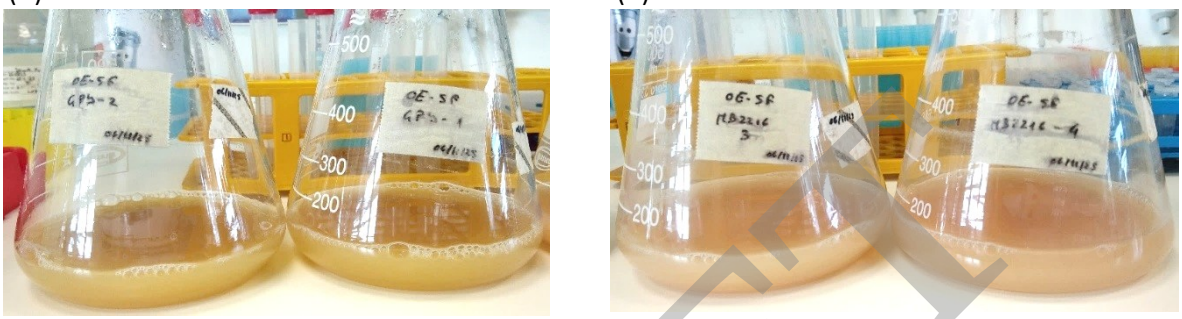

Figure 22 – Final fermentation broth obtained in S. frigidimarina growth study using different culture media: (A) rich medium; (B) marine broth.

According to these results, *Shewanella* spp. were chosen as the biocatalysers for next experimental steps taking into account the relevance of EPA among target PUFAs.

5.8 Test the growth of selected *Shewanella* spp. in mineral media enriched in commercial VFAs by a bench-scale fermenter

As mentioned above, the best productors of long chain PUFAs identified in *S. pacifica* DSM 15445 and *S. frigidimarina* DSM 12253 were grown in a Solaris model bioreactor, applying slightly different growth conditions; specifically, *S. pacifica* DSM 15445 was grown in 1.1 L of culture medium (as reported in Section 4.8) where 0.8 g/L NH_4Cl were used, while *S. frigidimarina* DSM 12253 was grown in a culture medium including 2.5 g/L of NH_4Cl, to apply conditions leading to major cellular biomass production.

Each culture employed around 48 hours for the glucose consumption and the addition of VFAs doses was executed manually when the preview dose was consummate. For each strain, about 3.5 g/L of VFAs were overall added. Both microorganisms were then harvested, washed and lyophilized for further analyses. 147 mg/L of lyophilized *S. pacifica* and 322 mg/L of lyophilized *S. frigidimarina* were obtained at the end of the experimental procedure. Both strains produced EPA, even if lower concentrations in the lipidic fraction were detected than those observed in the preliminary screening (Section 5.7). However, *S. frigidimarina* accumulated a significant amount of lipids (about 17% of the cell dry weight), so that the overall EPA concentration was slightly higher than that obtained by feeding the bacteria with glucose (Section 5.7). As the main consequence of such results, and taking into account the evidence collected by Biotrend (Section 5.7), *S. frigidimarina* was chosen as the biocatalyser for pursuing project aims concerning the production of target PUFAs. Accordingly, an exhausted characterization of the biomass collected after the fermentation process by centrifuging

the exhausted cultural broth was carried out by determining the centesimal biomass composition (Table 10). Results indicate that a high concentration of ashes was collected together with the microbial cells, due to a high occurrence of salts in the cultural medium: thus, further experiments were conducted to verify the feasibility of limiting the addition of salts in the preparation of the medium. Besides, a high amount of proteins were measured. The fatty acids composition of the *S. frigidimarina* biomass is reported in the following Table 11.

Table 10. Centesimal biomass composition (%) of biomass collected at the end of the fermentation experiment carried out with S. frigidimarina

Parameters (AOAC gravimetric methods, 2002*)	**Average %FW**
Moisture	7,98
DM	92,02
Ash-free DW (AFDW)	44,8
Total lipids (TLP)	9
Protein	45,2
Carbohydrates	-
Ashes	43,4
P (% of total ashes)	22,06

Table 11. Composition of the fatty acids fraction in the biomass of S. frigidimarina collected at the end of the fermentation experiment

GC-MS fatty acid profile (%total FAMES)	
C12:0 (lauric)	2.75 ± 0.65
C13:0 iso	1.93 ± 0.61
C13:0	2.24 ± 0.46
C14:0 (myristic)	12.62 ± 2.14
C14:1 isomers	1.01 ± 0.03
C15:0 anteiso	1.77 ± 0.03
C15:0	3.82 ± 0.72
C15:1 isomers	0.59 ± 0.14
C16:0 (palmitic)	16.73 ± 1.13
C16:1 sum of isomers	26.12 ± 5.35
C17:1 isomers	1.98 ± 0.27
C18:0 (stearic)	4.97 ± 0.73
C18:1 isomer 1	19.53 ± 11.12
cis-9 C18:1 (oleic)	1.09 ± 0.00
C18:2LA	1.46 ± 1.42
C20:0	0.53 ± 0.08
C20:5 (EPA)	0.86 ± 0.25
C22:6 (DHA)	-

A deep elemental characterization was also carried out in cooperation with UNIVPM (Table 12).

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Table 12. concentration of metals in the dry biomass of S. frigidimarina collected at the end of the fermentation process

Element	g/kg	Element	g/kg	Element	g/kg
As	-	Fe	25,35	Se	0,00
Be	-	Li	0,00	Ti	0,02
Ca	3,06	Mg	2,49	V	0,03
Cd	0,00	Mn	0,04	Zn	0,11
Co	0,00	Mo	0,00	Na	122,51
Cr	0,18	Ni	0,03	K	11,05
Cu	0,05	Pb	0,01	Si	0,10
				Al	1,66

As a whole, data concerning biomass composition represent key information for the formulation of fish feed, in the perspective of utilizing the whole biomass as an ingredient instead of extracted oil, this allowing the set-up of a more sustainable and cheap value chain.

5.9 Optimization of the mineral medium to be employed for the accumulation of target PUFAs by the selected *Shewanella frigidimarina* strain

According to observations related to the growth of commercial strains that were selected due to their potential capability of accumulating PUFAs (Sections 5.7 and 5.8), *S. frigidimarina* was chosen as the bacterium to be used in the process development, and efforts were carried out to improve the medium composition in order to avoid: i) salt precipitation; ii) unproper utilization of chemicals; iii) and consumption of alternative carbon sources (available in peptone and yeast extract) instead of VFAs. First experiments were dedicated to study the order of reagent addition for preparing the medium, namely: MgCl, CaCl, trace element solution and Fe-citrate. Hence, the precipitation was minimised but not completely avoided. Thereafter, the possibility of replacing Fe-citrate with Fe-EDTA to get a clear solution was assessed. Thus, experiments were set up in order to verify the growth of *S. frigidimarina* in the modified medium (Section 4.9).

Importantly, a significant and encouraging microbial growth was obtained, together with a satisfactory production of lipids and EPA in particular (Table 13); as a matter of fact, the content of total lipids was about 20% of the dry biomass, 1.22% of which constituted by EPA.

The simultaneous removal of peptone and yeast extract led to a lower lipids and EPA production, both by using Fe-citrate and Fe-EDTA, this demonstrating that their employment cannot be completely avoided; however, importantly, the latter Fe carrier allowed better performance (Table 13). These results are of a high interest in the perspective of optimizing the medium composition.

Table 13. Fatty acids production by medium with Fe-EDTA or Fe-citrate, in the absence (column "no Pep and YE") or in the presence of peptone and yeast extract

	Fe-EDTA no Pep no YE	Fe-citrate no Pep no YE	Fe-EDTA
TLP (%DM)	12,92	8,43	20,16
FAMES (rel %)			
C12:0 (lauric)	1,05	1,52	0,80
C13:0 iso	2,59	2,48	6,24
C13:0	0,43	0,61	0,42
C14:0 (anteiso)	0,51	0,15	0,59
C14:0 (iso)	0,39	0,13	-
C14:0 (myristic)	8,66	9,93	8,16
C14:1 isomers	0,64	0,76	1,02
C15:0 anteiso	2,45	1,83	7,65
C15:0 iso	0,13	0,10	0,37
C15:0	1,26	1,28	2,10
C15:1 isomers	0,14	0,15	0,39
C16:0 (palmitic)	26,06	27,50	19,16
C16:1 sum of isomers	39,57	37,59	35,97
C17:0 (anteiso)	0,27	0,18	0,54
C17:0	0,49	0,57	0,78
C17:1 isomers	1,19	1,13	2,12
C18:0 (stearic)	2,97	3,25	2,45
C18:1 isomer 1	4,14	3,65	3,79
cis-9 C18:1 (oleic)	5,69	6,26	5,84
C18:1 isomer 2	0,34	-	-
C18:2 LA	-	0,17	0,10
C18:3 ALA	-	-	-
C18:4 omega3	0,17	0,12	0,29
C20:0	0,10	0,10	-
C20:5 (EPA)	**0,77**	**0,54**	**1,22**
∑omega3	**0,95**	**0,66**	**1,50**

Thus, a further investigation was set up as described in Section 4.9 to verify if the employment of peptone and yeast extract can be lowered or avoided. Importantly, results indicate that the addition of both substrates can be limited by one order or magnitude (Table 14), since similar results were obtained with respect to those related to the growth of *S. frigidimarina* with Fe-EDTA (Table 13) in the occurrence of conventional amounts of both substrates.

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Table 14. Fatty acids production by medium with 10% of peptone (Pep) (0.5 g/L), b) 10% yeast extract (YE) (0.1 g/L) and c) 10% of both (0.5 g/L peptone + 0.1 g/L yeast extract)

	Pep 10%	YE 10%	Pep and YE 10%
TLP (%DM)	13,70	15,63	14,10
FAMES (rel %)			
C12:0 (lauric)	0,60	0,90	0,69
C13:0 iso	2,47	2,58	2,91
C13:0	0,54	0,41	0,69
C14:0 (anteiso)	0,13	0,24	0,35
C14:0 (iso)	0,00	0,21	0,28
C14:0 (myristic)	6,69	7,47	7,20
C14:1 isomers	0,59	0,81	0,54
C15:0 anteiso	3,06	3,28	3,89
C15:0 iso	0,40	0,37	0,52
C15:0	2,68	1,96	3,51
C15:1 isomers	0,30	0,21	0,38
C16:0 (palmitic)	22,16	23,57	23,02
C16:1 sum of isomers	33,62	35,11	34,54
C17:0 (anteiso)	0,44	0,33	0,36
C17:0	1,02	0,70	1,34
C17:1 isomers	1,62	1,42	2,17
C18:0 (stearic)	3,43	3,02	3,04
C18:1 isomer 1	9,58	7,79	5,52
cis-9 C18:1 (oleic)	5,72	6,14	6,31
C18:1 isomer 2	0,00	0,00	0,27
C18:2 LA	3,49	2,11	1,13
C18:3 ALA	0,38	0,24	0,16
C18:4 w3	0,13	0,19	0,14
C20:0	0,14	0,10	0,14
C20:5 (EPA)	**0,82**	**0,85**	**0,89**
∑omega3	**1,33**	**1,27**	**1,18**

Finally, another experiment was addressed to verify the possibility of lowering the concentration of salts in the medium; to this aim, a modified medium was prepared by halving the dose of each salt, with the exception of N-NH_4 salt (NH_4Cl) to assure a non-limiting availability of N that would result in an ineffective growth condition. Peptone was amended at 0.5 mg/L (10% of the conventional concentration in the MB514 medium), while no yeast extract was used. Despite the data for the overall lipid concentration is not available, a satisfactory growth was observed, and a slightly higher EPA production (1.10% of the lipidic fraction) was measured if compared with data obtained in previous experiments (Tabs. 13 and 14).

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5.10 Test the growth of selected *Shewanella frigidamarina* in a mineral medium enriched in VFA-rich fermented cheese whey

The last experiment carried out in the framework of Subtask 2.2.1 demonstrated that S. *frigidimarina* is able to uptake VFAs occurring in an actual fermentation broth, at the same concentration by which the solution with commercial acids was prepared to conduct preliminary tests in a bench-scale fermenter (Section 5.8). A significant amount of lipids were produced (about 12% of the overall dry matter), and EPA represented 0.8% of such a fraction (Table 15). This represent a crucial evidence in the perspective of developing and scaling-up a PUFA production process fed with the project target raw material.

Table 15. Fatty acids production by medium amended by spikes of an actual VFA-rich effluent obtained by processing a cheese whey under acidogenic fermentation conditions.

TLP (%DM)	12,10
FAMES (rel %)	
C12:0 (lauric)	0,88
C13:0 iso	2,58
C13:0	1,47
C14:0 (anteiso)	0,40
C14:0 (iso)	0,00
C14:0 (myristic)	5,85
C14:1 isomers	1,23
C15:0 anteiso	2,76
C15:0 iso	0,31
C15:0	5,54
C15:1 isomers	2,36
C16:0 (palmitic)	19,62
C16:1 sum of isomers	34,69
C17:0 (anteiso)	0,27
C17:0	1,25
C17:1 isomers	5,23
C18:0 (stearic)	4,49
C18:1 isomer 1	5,26
cis-9 C18:1 (oleic)	3,12
C18:1 isomer 2	0,23
C18:2 LA	1,31
C18:3 ALA	0,26
C18:4 w3	0,00
C20:0	0,10
C20:5 (EPA)	0,80
∑omega3	**1,06**

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5.11 Challenges encountered

During the implementation of Subtask 2.2.1, several challenges were encountered. One of the main difficulties was related to the variability of the VFA composition in the whey substrates provided by UNIVPM. The concentration and distribution of volatile fatty acids (acetic, propionic, and butyric acids in particular) changed according to the fermentation type and duration, influencing the reproducibility of yeast growth and lipid accumulation. This variability made it difficult to establish fully standardised experimental conditions and required repeated adaptation of the culture protocols.

In addition, the complex microbial ecosystem of fermented whey posed challenges in terms of contamination and metabolic competition. Indeed, after microfiltration (0.45 µm), the whey contained residual microbial activities capable of modifying the VFA profile during incubation. This phenomenon was particularly evident in static conditions, where an unexpected increase in butyric acid suggested that other microbial metabolisms were active. Although such behaviour is common in non-sterile systems, it prevented the full attribution of the observed metabolic changes solely to the yeast. It should also be noted that filtration at 0.2 µm, which would ensure higher sterility, is technically demanding and economically unfeasible at industrial scale.

One critical challenge was the identification and selection of bacterial strains capable of accumulating target PUFAs. In fact, despite preliminary evidence belonging to UNIBO background, marine bacteria previously isolated by UNIBO [15] were not observed to accumulate PUFAs, and in particular EPA and DHA. Thus, in agreement with the project contingency plan, a wide range of strains were screened both in silico and in vivo, by taking advantage of the bacterial collection available by UNIPR; however, only identified and acquired commercial microorganisms were observed to produce PUFAs.

Main challenges encountered for the cultivation of PUFA accumulating bacteria have concerned the formulation and preparation of a culture medium, which not undergoes salt precipitation. Several experiments have been designed and carried out to find out a feasible protocol for an improved cultivation of identified marine bacteria, by limiting the employment of salts and simultaneously their occurrence in dried biomass, thus improving the concentration of PUFA and organic fractions when the whole dry biomass is used for the formulation of animal feeds.

Despite these challenges, the experiments presented in this deliverable successfully demonstrated: a) the metabolic versatility of *Y. lipolytica,* and established a solid foundation for future scale-up activities under Task 2.2.2; b) the possibility of feeding the microorganisms employed in this research with actual VFA-rich acidogenic fermentation broths, and the capability of such strains of uptaking VFAs for the biosynthesis of PUFAs; c) the possibility of improving the formulation of culture media, by limiting the employment of chemicals and obtaining final biomasses less contaminated by inorganic components that lower the relative amount of target organic materials, such as PUFAs.

6 Main Findings and Conclusion

Subtask 2.2.1 confirmed the technical feasibility of converting VFA-rich dairy effluents into microbial oils and biomasses enriched in PUFAs through the use of *Yarrowia lipolytica* and *Shewanella frigidimarina*, respectively.

Among the ten yeast strains initially screened, Y3, RO2, and RO3 showed the best growth performance in minimal medium containing acetic, propionic, and butyric acids. Among these, *Y. lipolytica* Y3 was selected as the most promising candidate due to its balanced growth, tolerance to SCFAs, and ability to accumulate unsaturated fatty acids. The strain was then tested in VFA-enriched minimal media and real cheese whey substrates produced by UNIVPM. Growth was strongly influenced by the substrate composition and incubation mode. Natural-fermented whey proved to be the most suitable medium, supporting cell densities up to 8 log CFU/mL and promoting a balanced accumulation of biomass and lipids. In contrast, kefir- and *Propionibacterium*-fermented whey showed lower growth performance and lipid yields, confirming the strong dependency of *Y. lipolytica* metabolism on substrate origin and acid composition. Aeration was found to stimulate metabolic activity and accelerate the consumption of VFAs, but it primarily favoured biomass production rather than lipid accumulation. Static incubation, instead, led to higher lipid content, reaching up to 42% of CDW after 13 days in Squacquerone whey, and 2.7% in short 3-day fermentations with sterile natural-fermented whey. Fatty acid analysis revealed a profile dominated by long-chain unsaturated fatty acids, mainly C18:1, C18:2, and C18:3, which accounted for more than 80% of total lipids. Although true PUFAs were not detected, the strong enrichment in monounsaturated and polyunsaturated fatty acids highlights the high nutritional and industrial potential of the obtained microbial oil.

The large screening of bacteria potentially capable of producing PUFAs allowed to define the microbial strain (*Shewanella frigidimarina*), which will be employed in the process scale-up. The strain was able to utilize an actual fermentation broth enriched in VFAs as a substrate for the accumulation of PUFAs. In particular, lipids constituted about 12% of the overall dry biomass, and EPA represented about 1% (w/w) of such a fraction: this result is promising in the perspective of further optimizing the production of PUFAs from VFAs occurring in effluents of anaerobic acidogenic processes fed with organic residues, such as cheese whey. A deep characterization was performed of the biomass produced by feeding *Shewanella frigidimarina* with VFAs in a bench-scale fermenter: although lower amounts of overall lipids and EPA were obtained, the employment of the fermenter allowed to demonstrate the feasibility of the process scale-up and to collect a critical biomass amount for performing the biomass analyses. This activity allowed to understand the importance of lowering the addition of salts in the medium formulation, so to minimize the final content of ashes: notably, about 43% of the solids collected from the reactor were represented by ashes (inorganic compounds). As a matter of fact, the activity concerning the optimization of the culture medium demonstrated that an improved solubilisation of medium components can be achieved by a) replacing Fe-citrate with Fe-EDTA and b) reducing of 50% the concentration of salts, except for that of the ammonium salt; furthermore, an effective medium can be formulated without the addition of yeast extract and by amending peptone at a concentration of one order of magnitude lower than that of the conventional MB514 medium. As a whole, this evidence is of interest in the perspective of limiting the requirement of chemicals for the cultivation of the *S. frigidimarina* strain.

From an applied perspective, these findings confirm that fermented dairy by-products can serve as low-cost and sustainable feedstocks for the production of biomass enriched in PUFAs and lipids. The results obtained at TRL 3 provide a knowledge base for pilot-scale validation in Subtask 2.2.2. Future work will aim to further optimize the fermentation parameters and fine-tune oxygen availability to enhance total lipid yield and PUFA content. More in details, further efforts will be targeted at cultivating the *Shewanella frigidimarina* employed in the experiments in bench scale fermenters, fed with an optimized medium and actual acidogenic effluents as the source of VFAs.

In conclusion, the study provides a convincing proof of concept for integrating *Y. lipolytica* and *Shewanella frigidimarina* into circular bioeconomy strategies, turning VFA-rich whey effluents into value-added microbial oils suitable for applications in food, feed, and bio-based materials.

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