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Selection of *Yarrowia lipolytica* Strains as Possible Solution to Valorize Untreated Cheese Whey

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Abstract: Cheese whey management and disposal is a major issue for dairy industries due to its high level of chemical and biochemical oxygen demand. However, it can still represent a source of nutrients (i.e., sugars, proteins and lipids) that can be applied, among other options, as substrate for microbial growth. *Yarrowia lipolytica* can grow in different environments, consuming both hydrophilic and hydrophobic substrates, and tolerates high salt concentrations. In this work, the lipolytic and proteolytic profile of 20 strains of *Y. lipolytica* were tested on caseins and butter. Then, their growth potential was evaluated in four types of whey (caciotta, ricotta, squacquerone and their mix). *Y. lipolytica* showed a very strain-dependent behavior for both hydrolytic profiles and growth capabilities on the different substrates. The best growers for all the types of whey tested were PO1, PO2, and RO2, with the first one reaching up to 8.77 log cfu/mL in caciotta whey after 72 h. The volatile molecule profile of the samples incubated with the best growers were characterized by higher amounts of esters, acids, ketones and alcohols. In this way, cheese whey can become a source of microbial cultures exploitable in the dairy sector.

Keywords: *Yarrowia lipolytica*; cheese whey valorization; dairy waste; proteolytic activity; lipolytic activity

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1. Introduction

Yarrowia lipolytica is one of the most extensively studied yeast species, after *Saccharomyces cerevisiae*. It has been isolated from several food matrices (i.e., yoghurt, kefir, soy sauce, rancid margarine, shrimp salads, sourdough) and different environments (soil, oil-polluted soil, rivers and sea water) [1]. Although it has been referred to as a spoilage yeast, due to its strong proteolytic and lipolytic activity, *Y. lipolytica* is fundamental for the ripening of some traditional dry fermented sausages and cheeses, where it can represent the dominant microorganism [2]. It is classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (FDA) and recommended by the European Food and Safety Authority (EFSA) as having qualified presumption of safety (QPS), but only for production purposes [3–5]. However, the same European authority provided inactivated *Y. lipolytica* biomass with the status of a novel food, that can be consumed as a supplement by the general population from three years of age onwards. In fact, these biomasses could be an important source of proteins, dietary fiber, and fats [6]. The capability to grow on different types of substrates (carbohydrates, lipids, and proteins) and to tolerate high levels of NaCl, makes this yeast a promising candidate to be used for food waste and by-products valorization [1].

Cheese whey (CW) is the co-product of cheesemaking. From each kg of cheese produced, around 9–10 L of whey are obtained. Production of CW is estimated at around 190 million tons/year worldwide [7]. CW contains mainly lactose, proteins, lipids, mineral

salts, and other minor components (e.g., lactic acid, citric acid, urea and type-B vitamins) [8]. Their concentrations depend on the milk's origin and the process applied to make the cheese. Due to its composition, CW has high biological (BOD_5) and chemical oxygen demand (COD), ranging from 30 to 50 and 60 to 80 g/L, respectively. For this reason, its disposal represents a serious issue from both an economical and an environmental point of view [9]. Part of CW is already reused in the dairy industry to make whey-cheeses and whey-butter, which, in turn, generates the so-called secondary cheese whey. Alternatively, protein and lactose can be extracted and used as ingredients in dairy and bakery products, or in animal feed (although the high lactose level may induce digestive disorders in animals) [10,11]. The remaining unused whey is applied as biofertilizer [12]. However, this is not a sustainable approach and can cause risk for fields. Therefore, finding alternative applications is always desired. For instance, biotechnological approaches can be used to obtain prebiotics, organic acids, enzymes, single cell proteins (SCPs), and biodiesel [11–16]. As applied in the context of the European project INGREEN, three main routes can be foreseen. The first one exploits enzymes (e.g., β-galactosidase to produce galactooligosaccharides, [17]), the second one uses microorganisms to produce novel compounds (e.g., *Pseudomonas taetrolens* strains to convert lactose into lactobionic acid, [18]), and the last one uses microorganisms to obtain microbial biomasses that can be reused in different fields (as a source of proteins [19], starter or adjunct cultures [20,21]). The selection of enzymes, or microorganisms, that are applied on CW is crucial and defines the final ingredients generated.

Studies regarding the application of *Y. lipolytica* in dairy by-products are not numerous, maybe due to the complexity and variability of the substrates, but also because the yeast should not assimilate lactose as a carbon source [22]. However, Taskin et al. [23] isolated the lactose-positive strain B9, meaning that this feature is strain dependent and other isolates may possess the same attitude. Moreover, most of the studies focused on efficient production of valuable metabolites, such as single cell oils and citric acid, that require high carbon/nitrogen ratios. Since lactose should not be assimilated, the C/N ratio of CW is not favorable for metabolite production. Therefore, other solutions were adopted, such as simple sugar supplementation [24] or use of deproteinized whey [25]. However, as already mentioned above, *Y. lipolytica* can grow on substrates that are rich in fats or proteins, such as cheeses and meat [26–28].

The aim of this study was to screen 20 strains of *Y. lipolytica*, isolated from different environments and characterized in previous studies [27,29–31], for their possible application as food adjuncts in cheesemaking. In particular, their enzymatic capacities were assessed by monitoring their proteolysis and lipolysis profiles in vitro. To make the production of the new adjunct more sustainable, yeast growth was evaluated in four different dairy wastes used as microbial substrates. Eventually, the samples obtained with the most promising strains were studied for their volatile molecule profiles to assess the presence of undesired compounds.

2. Materials and Methods

2.1. Microbial Cultivation

The 20 strains of *Yarrowia lipolytica* belong to the Department of Agricultural and Food Sciences of the University of Bologna and were isolated through the years from different environments: Po River (PO1, PO10, PO19, PO2, PO20, PO23), dairy products (RO1, RO2, RO20, RO21, RO22, RO7, RO8, RO9), refrigerated food (Y2, Y20, Y21, Y22, Y5, Y6). Theses strains were characterized and applied in previous studies [26,27,29,31]. The yeasts were grown at 25 °C for 72 h in Yeast Peptone Dexstrose (YPD) (Glucose 20 g/L; Universal peptone 10 g/L; Yeast extract 10 g/L) prior to their use for all the trials. The ITS regions of the three most promising strains (PO1, PO2 and RO2) were amplified with the primers ITS1 and ITS4. The amplicons were subjected to Sanger's sequencing and the obtained sequences were aligned to the rRNA/ITS databases of the NCBI, using BLAST as the alignment tool. The sequences of the strains identified as *Yarrowia lipolytica* were submitted to the NCBI

GenBank database with the following accession numbers: OQ103350, OQ103351, and OQ103352, respectively.

2.2. Cheese Whey

Samples of whey used in this work were provided by Mambelli srl (Bertinoro, FC, Italy). The following four types of whey were considered: (1) caciotta whey, obtained from caciotta cheesemaking, (2) squacquerone whey, obtained from squacquerone production, (3) ricotta whey, obtained from ricotta production, (4) mixed whey, obtained from different wheys stored in non-refrigerated conditions for 1–2 days. After production, the different types of whey were stored at −20 °C, for their nutritional characterization, or immediately collected in sterile containers and used as substrates for *Y. lipolytica*.

2.3. Proteolysis Profile

The proteolysis profile was generated by incubating the supernatant of 72 h grown *Y. lipolytica* strains on α-casein or β-casein solution (final concentration 1 mg/mL), for 72 h at 25 °C. At the end of incubation, protein profiling was obtained using SDS–PAGE electrophoresis. A quantity of 70 μL of the samples were mixed with equal volumes of Laemmli Sample Buffer 2X (Bio-Rad Laboratories, Milan, Italy) containing β-mercaptoethanol. The mixtures were incubated at 100 °C for 10 min and then charged on an 8–16% Criterion TGX precast Gel (Bio-Rad Laboratories, Milan, Italy). Then, 10 μL of Precision Plus Protein Standard All Blue (Bio Rad) was used as standard, and 35 μL of each sample were loaded on the gel. Gel was run in a Mini Protean Cell System with a Tris-Glycine SDS Running Buffer at 100 V for the first 10 min and at 200 V for 1 h. Gels were stained for 1 h with the staining solution (0.1% Bromophenol blue, 50% methanol and 7% glacial acetic acid) and de-stained for 2 h with the de-staining solution (40% Glacial acetic acid, 10% methanol). Pictures were taken with Bio-Rad's GS-900 (Bio-Rad, Milan, Italy).

2.4. Lipolysis Profile

The lipolysis profile was generated as described by Guerzoni et al. [30]. Free fatty acid (FFA) analyses were performed, according to Burns et al. [32]. FFAs were separated using a Bond Elut aminopropyl column (3 mL, containing 500 mg of silica modified with aminopropyl groups, IST, Mid Glamorgan, UK) and eluted with 10 mL of diethyl ether containing 2% (vol/vol) formic acid. Quantification and identification of FFA was performed with a Gas-Chromatography (GC) 7890A coupled with mass spectrometry (MS) 5975C (Agilent Hewlett–Packard, Geneva, Switzerland) and a DB-5 (60 m × 0.25 i.d.) column (J&W, Agilent, Milan, Italy). The identifications of the individual FFAs were obtained by comparing the unknown peaks with those of an FFA standard (Sigma-Aldrich, Milan, Italy) and matching their mass spectral data with those of the compounds contained in the Agilent Hewlett-Packard NIST 11 mass spectral database.

2.5. Cheese Whey Characterization

Chemico-physical characterization was performed by Tecnal s.r.l. following national and international standard procedures (fats, MP13C/2016 Rev.10; proteins ISO14891:2002; lactose, Rapporti Istisan 1996/34; lactic acid, MP 87C; Total nitrogen, ISO14891:2002; BOD5, MP4D/2020 Rev.17; COD, ISO 15705:2002; metals and ions, UNI EN 13805:2014 + ISO 11885:2007; PO4, APAT CNR IRSA 4020 Man 29 2003). Microbial characterization was performed by plating the samples, and relative dilutions, in the following: Yeast extract, peptone, dextrose (YPD) agar for yeasts; deMan, Rogosa, and Sharpe (MRS) agar for lactic acid bacteria; and Violet Red Lactose Bile Agar (VRBGA) for Enterobacteriaceae. All the media were provided by Oxoid (Milan, Italy). Colonies were counted after 48 h at 30 °C for YPD, 48–72 h at 37 °C for MRS and 24 h at 37 °C for VRBGA.

2.6. Screening Y. lipolytica Growth in Cheese Whey

After two refreshments, broth culture from each strain of *Y. lipolytica* was transferred into sterile falcon tubes and centrifuged at 8000 rpm for 15 min at 20 °C. The pellet was washed with sterile saline solution (NaCl, 9 g/L), resuspended in the same solution, and used as an inoculum. Then, 300 mL of each waste were transferred into sterile Erlenmeyer flasks and inoculated with *Y. lipolytica*. Inoculated samples were incubated at 25 °C for 72 h. During this time, samples were collected at 24, 48 and 72 h to quantify *Y. lipolytica* by plate counting in YPD. The samples with the best growers (strain PO1, PO2 and RO2) were also characterized for LAB and Enterobacteriaceae contents and volatile molecules produced. The work was performed in triplicate for each strain and each whey tested (n = 3).

2.7. Volatile Molecule Profile

The molecule volatile profiles were detected by using the gas chromatography/mass spectrometry/solid phase microextraction (GC/MS/SPME) technique, as described by [9]. Briefly, 5 mL of each sample were split in vials and stored at −20 °C prior to analysis. A CAR/PDMS, 75 μm fiber (SUPELCO, Bellafonte, PA, USA) was used to perform SPME. The samples were incubated for 10 min at 45 °C. Then, the fiber was exposed to the vial headspace for 30 min at 45 °C. The volatile molecules adsorbed were desorbed in the GC injector port in splitless mode at 250 °C for 10 min. The headspace of the volatile compounds was analyzed using gas GC 7890A, combined with MS 5975C (Hewlett–Packard, Geneva, Switzerland). The column used was Chrompack CP-Wax 52 CB (50 m × 320 μm × 1.2 μm). The initial temperature was 40 °C for 1 min and was then increased by 4.5 °C/min up to 65 °C. After that, the temperature was increased by 10 °C/min up to 230 °C and remained at this temperature for 17 min. Compounds were identified by comparison based on the NIST 11 (National Institute of Standards and Technology) database. The gas-carrier was helium at a 1.0 mL/min flow. An internal standard (4-methyl-2-pentanol, final concentration 20 ppm) was added to each sample before SPME analyses and used to obtain the relative concentrations of the compounds.

2.8. Data Analyses

For each sample, the microbiological and volatile data were the mean of three different samples of three independent experiments. Principal component analysis (PCA) was performed using Statistica software (version 8.0; StatSoft., Tulsa, OK, USA) to obtain a visual overview of the volatile molecule profiles. Microbiological data were also analyzed with Statistica software. Means were compared using one way-ANOVA followed by Tukey HSD test at $p < 0.05$ level.

3. Results

3.1. Enzymatic Properties of the 20 Strains of Y. lipolytica

3.1.1. Proteolysis Profile

The proteolysis profile obtained with the 20 strains of *Y. lipolytica* grown on α-casein are shown in Figure 1. From an overall perspective, *Y. lipolytica* isolated from Po river (PO series) promoted the strongest hydrolysis, followed by the isolates from dairy products (RO series) and refrigerated food (Y series). However, a strain-specific profile of bands was also observed. PO2, PO20, and RO9 did not show any bands, while RO7, RO8, Y22, Y2 and Y6 presented bands with molecular weights (MWs) below 30 kDa. All the other strains performed mild proteolysis, since the native casein (34 kDa) was still partially present, together with the formation of low MW bands.

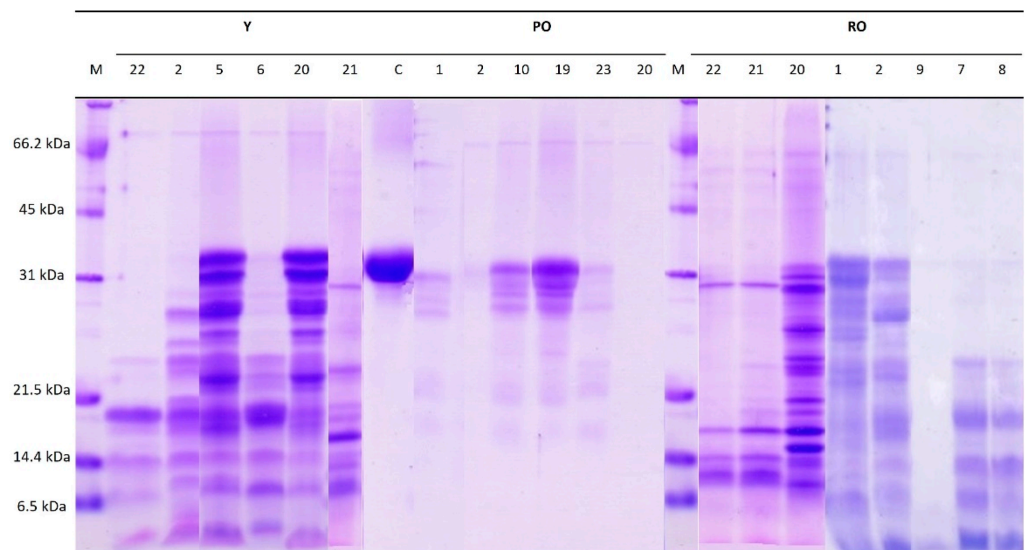

Figure 1. SDS–PAGE electrophoresis of α-casein hydrolyzed with different strains of *Y. lipolytica* after 72 h of incubation. C: α-casein alone (~34 kDa), M: Protein marker.

When the trial was repeated using β-casein as substrate, the overall trend was similar (Figure 2). In fact, PO and RO series showed the strongest hydrolysis, followed by the Y series. From a strain point of view, PO1, PO2, and PO10 did not show any bands, while Y2, Y22, Y6, RO2, RO9, RO7, and RO8 presented bands only equal to, or below, 14.4 kDa. Instead, milder activity, with a partial presence of the native β-casein bend, was observed for strains Y5, Y20, PO19, PO23, PO20, Y21, RO22, RO21, RO20 and RO1.

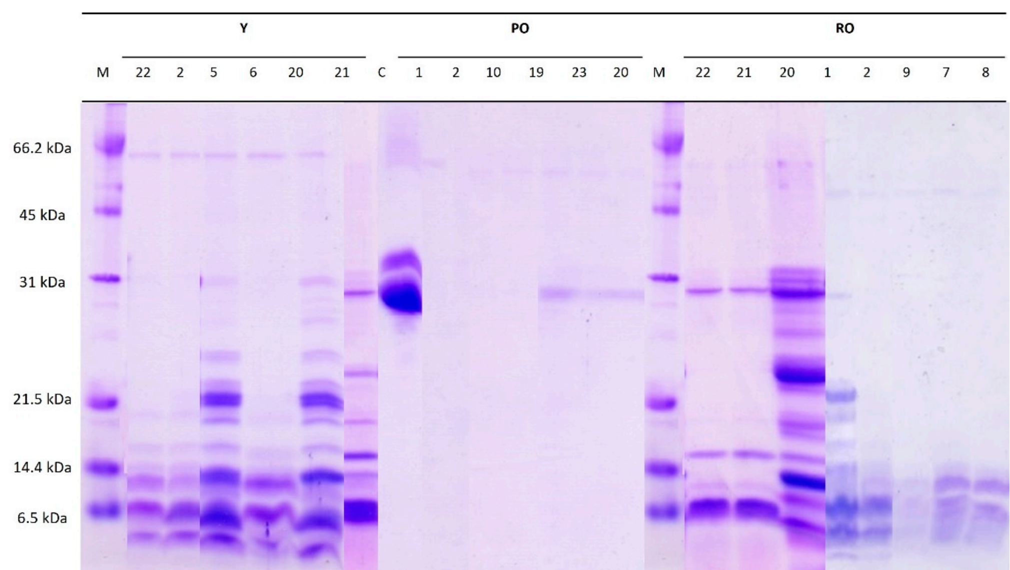

Figure 2. SDS–PAGE electrophoresis of β-casein hydrolyzed by different strains of *Y. lipolytica* after 72 h of incubation. C: β-casein alone (~31 kDa), M: Protein marker.

3.1.2. Lipolysis Profile

The butter used to test the lipolysis was mainly composed of saturated fatty acids, such as C16:0 (20.7%) and C18:0 (47.3%) (Figure 3). Addition of *Y. lipolytica* strains induced strain-specific modifications on FFAs' abundance. For instance, an increase in unsaturated FFAs was observed using strain PO1, PO20, PO23, RO21 and Y5. In fact, in these samples the unsaturation levels were above 60%. Among the unsaturated FFAs, the abundance of C18:1 increased in all the samples, whereas the presence of other strains determined an

increase in saturated medium chain fatty acids, such as C12:0 (RO1, RO9, RO20, PO23), C14:0 (mainly, PO1, PO2, PO20, PO23, RO7, RO8, RO22, Y2, Y5, Y6, Y20, Y22), and C16:0 (PO19, Y6, Y21).

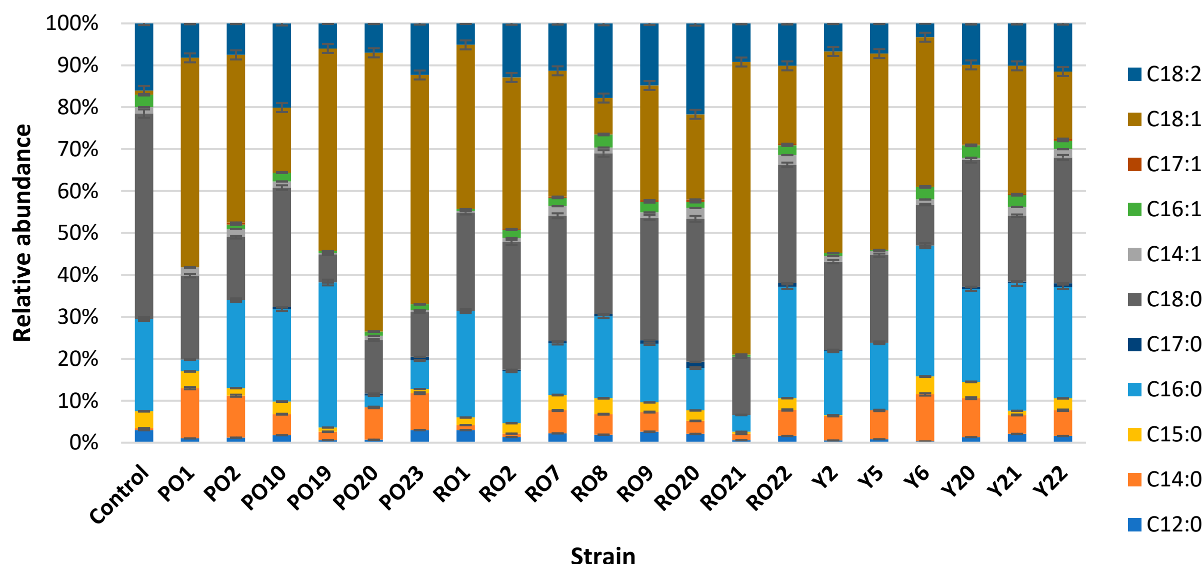

Figure 3. Relative abundance of FFAs released by different *Y. lipolytica* strains inoculated in butter samples. The values are the mean of three replicates and are expressed in percentage (%) with respect to the total area measured.

3.2. Growth in Cheese Whey

3.2.1. Cheese Whey Characterization

The four types of whey used in this work as substrates for *Y. lipolytica* were characterized from a chemical point of view. As it is possible to observe in Table 1, all the samples contained lactose (40–50 g/L) as the first macronutrient, followed by proteins (5.8–11.0 g/L), fats (3.0–5.0 g/L) and lactic acid (1.5–7.7 g/L). Squacquerone whey had the highest concentration of lactose and proteins compared to caciotta and ricotta whey. On the other hand, caciotta whey showed the highest amount of fats (5 g/L). Mixed whey had a higher lactic acid content (7.7 g/L) and a lower pH. In fact, while all the other samples presented a pH between 6.0 and 6.5, mixed whey had a pH of 3.6. A microbiological characterization before using the different types of whey as a substrate for *Y. lipolytica* showed that native lactic acid bacteria (LAB), coming from cheesemaking, were present in caciotta and squacquerone whey at concentrations of 3.5 $\pm$ 0.20, and 4.7 $\pm$ 0.12 log cfu/mL, respectively. This was not surprising since the first cheese was made with a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgarigus*, while squacquerone was made with a blend of *S. thermophilus.* On the other hand, LAB were below the detection limit in ricotta whey. In fact, the product underwent a thermal treatment during production and no starters were added. Enterobacteriaceae were below the detection limit in all the samples. Eventually, indigenous yeasts were observed only in caciotta whey (1.5 $\pm$ 0.10 log cfu/mL), while yeasts were below the detection limit in all the other types of whey.

3.2.2. Screening *Y. lipolytica* Growth in Cheese Whey

Y. lipolytica growth was monitored at 24, 48 and 72 h in all the dairy by-products. While some strains poorly grew in caciotta whey (i.e., strain PO23, RO20, RO22, Y20, Y21 and Y22), all the others showed an increase in cell count (Table 2). Discarding the first ones, an average increase of 1.3 log cfu/mL was already estimated after 24 h, with a subsequent increase of 0.5 and 0.6 log cfu/mL in the following 48 and 72 h, respectively. The best growers in caciotta whey were PO1, PO2, and RO7, reaching 8 log cfu/mL after 72 h, followed by RO2, RO8, RO9, Y5 and Y6 (above 7 log cfu/mL). Looking at the overall pH

values over time (Table 2), decreases of 0.3, 1.3, and 0.34 were determined after 24, 48 and 72 h. Except for samples inoculated with RO1 and RO2, all the other pHs reached values below 5 after 72 h, with the lowest ones (pH 4.1 and 4.2) observed in samples containing RO9 and PO23.

Table 1. Physio-chemical composition of the different cheese wheys used in this study. The data are the mean of three independent measurements. (n = 3). Different letters among the samples mean significant differences ($p < 0.05$).

Parameters	Measuring Unit	Caciotta Whey	Ricotta Whey	Squacquerone Whey	Mixed Whey
Fats	g/L	4.7 ± 1.5 a	3.4 ± 0.3 a	2.9 ± 0.1 b	3.5 ± 2.1 a
Proteins	g/L	10.7 ± 1.5 a	6.0 ± 0.1 b	9.9 ± 1.9 a	11.0 ± 0.9 a
Lactose	g/L	46.9 ± 0.8 a	45.8 ± 1.5 a	48.8 ± 0.9 b	40.5 ± 0.2 c
Glucose	g/L	1.3 ± 0.5 a	<0.1 b	2.0 ± 0.8 a	<0.1 b
Lactic acid	g/L	1.5 ± 0.8 a	1.2 ± 1.1 a	1.3 ± 0.5 a	7.7 ± 0.1 b
Chemical Oxygen Demand (COD)	g/L O_2	90.9 ± 4.2	78.6 ± 14.9	84.7 ± 3.8	81.1 ± 14.0
Total nitrogen	%	0.17 ± 0.03	0.10 ± 0.02	0.16 ± 0.03	0.18 ± 0.01
pH		6.5 ± 0.06 a	6.0 ± 0.07 a	6.5 ± 0.11 a	3.6 ± 0.11 b
Phosphorous	mg/kg	450 ± 52	351 ± 82	448 ± 46	421 ± 18
Phosphates as PO_4	mg/kg	1379 ± 158	1075 ± 249	1373 ± 141	1291 ± 56
Calcium	mg/kg	380 ± 64	354 ± 72	361 ± 129	328 ± 18
Potassium	mg/kg	1450 ± 145	1428 ± 92	1451 ± 151	1449 ± 62
Sodium	mg/kg	3386 ± 332	2588 ± 356	2672 ± 350	2913 ± 164
Iodine	mg/kg	0.48 ± 0.10	3.02 ± 0.36	0.47 ± 0.14	-

Table 2. Concentrations of *Y. lipolytica* in caciotta whey and pH over time. Results are the mean of three different measurements. Different letters mean significant differences ($p < 0.05$) within a timepoint.

	Caciotta Whey							
	Concentration (log CFU/g)				pH			
Strain	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
PO1	4.9 ± 0.2	6.5 ^{m} ± 0.06	7.1 ^{m,n} ± 0.01	8.8 ^{i} ± 0.15	6.5 ± 0.1	6.4 ^{i,l} ± 0.02	5.2 ^{g} ± 0.01	4.2 ^{a,b,c} ± 0.02
PO10	4.9 ± 0.2	6.0 ^{f,g,h} ± 0.13	6.1 ^{e,f} ± 0.02	6.5 ^{c,d,e} ± 0.12	6.5 ± 0.1	6.5 ^{l} ± 0.03	5.1 ^{f} ± 0.02	4.3 ^{a,b,c} ± 0.01
PO19	4.9 ± 0.2	6.1 ^{f,g,h,i} ± 0.08	6.6 ^{g,h,i,l} ± 0.09	6.3 ^{b,c,d} ± 0.04	6.5 ± 0.1	6.4 ^{h,i} ± 0.01	5.1 ^{e,f} ± 0.05	4.8 ^{f} ± 0.01
PO2	4.9 ± 0.2	6.3 ^{i,l} ± 0.18	6.8 ^{h,i,l,m} ± 0.06	8.6 ^{i} ± 0.03	6.5 ± 0.1	6.4 ^{i,l} ± 0.05	5.5 ^{h} ± 0.01	4.5 ^{d,e} ± 0.01
PO20	4.9 ± 0.2	6.1 ^{g,h,i} ± 0.11	6.4 ^{f,g,h} ± 0.10	6.5 ^{c,d} ± 0.01	6.5 ± 0.1	6.0 ^{c} ± 0.01	4.5 ^{a} ± 0.03	4.5 ^{d} ± 0.02
PO23	4.9 ± 0.2	5.0 ^{c} ± 0.01	5.1 ^{b} ± 0.13	5.0 ^{a} ± 0.01	6.5 ± 0.1	6.3 ^{f,g} ± 0.01	4.5 ^{a} ± 0.01	4.2 ^{a} ± 0.02
RO1	4.9 ± 0.2	6.2 ^{h,i} ± 0.06	6.9 ^{i,l,m} ± 0.12	6.7 ^{d,e,f} ± 0.05	6.5 ± 0.1	5.9 ^{b} ± 0.02	5.0 ^{e} ± 0.03	5.3 ^{i,l} ± 0.23
RO2	4.9 ± 0.2	6.9 ^{a} ± 0.01	7.3 ^{n} ± 0.25	7.5 ^{f} ± 0.10	6.5 ± 0.1	5.6 ^{a} ± 0.03	5.2 ^{g} ± 0.01	5.4 ^{l} ± 0.12
RO20	4.9 ± 0.2	5.0 ^{c} ± 0.01	5.3 ^{b,c} ± 0.04	5.0 ^{a} ± 0.01	6.5 ± 0.1	6.1 ^{c,d} ± 0.04	4.7 ^{b} ± 0.02	4.6 ^{d,e} ± 0.03
RO21	4.9 ± 0.2	5.8 ^{e} ± 0.02	6.3 ^{e,f,g} ± 0.03	6.2 ^{b,c} ± 0.01	6.5 ± 0.1	6.0 ^{c} ± 0.06	4.7 ^{b,c} ± 0.02	4.4 ^{b,c,d} ± 0.01
RO22	4.9 ± 0.2	4.6 ^{b} ± 0.01	4.0 ^{a} ± 0.26	5.0 ^{a} ± 0.01	6.5 ± 0.1	6.2 ^{d,e} ± 0.04	4.8 ^{d} ± 0.01	4.3 ^{a,b,c} ± 0.01
RO7	4.9 ± 0.2	5.9 ^{e,f,g} ± 0.11	6.4 ^{f,g,h} ± 0.16	8.3 ^{h} ± 0.12	6.5 ± 0.1	6.5 ^{l} ± 0.01	5.0 ^{e} ± 0.04	4.4 ^{c,d} ± 0.01
RO8	4.9 ± 0.2	6.0 ^{e,f,g,h} ± 0.01	6.5 ^{f,g,h,i} ± 0.18	7.7 ^{g} ± 0.19	6.5 ± 0.1	6.4 ^{i,l} ± 0.01	5.3 ^{g} ± 0.01	4.2 ^{a,b} ± 0.05
RO9	4.9 ± 0.2	6.1 ^{g,h,i} ± 0.04	6.6 ^{g,h,i,l} ± 0.03	7.1 ^{g} ± 0.02	6.5 ± 0.1	6.4 ^{i,l} ± 0.05	4.7 ^{b,c} ± 0.03	4.1 ^{a} ± 0.01
Y2	4.9 ± 0.2	6.4 ^{l,m} ± 0.07	6.9 ^{l,m} ± 0.08	6.8 ^{f} ± 0.01	6.5 ± 0.1	6.4 ^{i,l} ± 0.02	5.0 ^{e} ± 0.01	4.8 ^{f,g} ± 0.01
Y20	4.9 ± 0.2	4.6 ^{b} ± 0.05	5.0 ^{b} ± 0.02	5.0 ^{a} ± 0.01	6.5 ± 0.1	6.3 ^{g,h} ± 0.02	4.8 ^{d} ± 0.01	5.0 ^{g,h} ± 0.02
Y21	4.9 ± 0.2	5.4 ^{d} ± 0.01	5.7 ^{c,d} ± 0.18	5.0 ^{a} ± 0.02	6.5 ± 0.1	6.2 ^{e,f} ± 0.01	4.8 ^{c,d} ± 0.04	4.8 ^{f,g} ± 0.02
Y22	4.9 ± 0.2	5.9 ^{e,f} ± 0.02	6.0 ^{d,e} ± 0.07	5.8 ^{b} ± 0.01	6.5 ± 0.1	6.1 ^{d} ± 0.01	4.6 ^{b} ± 0.05	4.8 ^{f,g} ± 0.01
Y5	4.9 ± 0.2	6.4 ^{l,m} ± 0.03	6.8 ^{h,i,l,m} ± 0.18	7.0 ^{g} ± 0.02	6.5 ± 0.1	6.4 ^{i,l} ± 0.03	5.3 ^{g} ± 0.01	4.7 ^{e,f} ± 0.01
Y6	4.9 ± 0.2	6.4 ^{l,m} ± 0.01	6.6 ^{g,h,i,l} ± 0.13	7.5 ^{f,g} ± 0.07	6.5 ± 0.1	6.5 ^{l} ± 0.04	5.6 ^{i} ± 0.01	5.1 ^{h,i} ± 0.01

The results of incubation performed in ricotta whey are reported in Table 3. After 72 h incubation, almost all the strains grew, except for RO1 and RO22. The average increases (without the values of the poor growers) were 1.3, 0.4 and 0.4 log cfu/mL after 24, 48 and 72 h, respectively. The best growers in ricotta whey were PO2 and PO20, that reached a concentration of 7.9 log cfu/mL after 72 h. These strains were followed by PO1, PO19, RO2, RO21, RO8, RO9, Y21, Y22, Y5 and Y6, with at least 6.9 log cfu/mL. The mean pH remained quite stable at 24 h, while it reduced after 48 h, particularly in the samples with RO1 and RO2. After 72 h, most of the samples had a pH below 5, while some (PO1, PO10, PO2, PO20, RO8) showed a pH increase above 6.5.

Table 3. Concentrations of *Y. lipolytica* in ricotta whey and pH over time. Results are the mean of three different measurements. Different letters mean significant differences ($p < 0.05$) within a timepoint.

	Ricotta Whey							
	Concentration (log CFU/g)				pH			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
PO1	4.9 ± 0.2	6.2 ^{e,f,g,h} ± 0.01	6.9 ^{f,g} ± 0.16	7.3 ^{f,g,h} ± 0.03	6.0 ± 0.07	6.0 ^{f,g} ± 0.02	5.9 ^{g,h} ± 0.02	7.0 ^{l} ± 0.01
PO10	4.9 ± 0.2	5.8 ^{d,e} ± 0.01	6.3 ^{c,d} ± 0.02	6.0 ^{b} ± 0.01	6.0 ± 0.07	6.0 ^{f,g} ± 0.01	6.2 ^{m,n} ± 0.06	6.7 ^{i} ± 0.01
PO19	4.9 ± 0.2	7.0 ^{i} ± 0.02	7.4 ^{h} ± 0.01	7.7 ^{i} ± 0.01	6.0 ± 0.07	6.1 ^{i} ± 0.04	6.2 ^{l,m} ± 0.01	5.9 ^{f,g} ± 0.05
PO2	4.9 ± 0.2	6.4 ^{g,h} ± 0.10	7.0 ^{f,g} ± 0.19	7.9 ^{i} ± 0.08	6.0 ± 0.07	6.1 ^{i} ± 0.01	6.2 ^{l,m} ± 0.01	7.0 ^{l} ± 0.11
PO20	4.9 ± 0.2	6.9 ^{i} ± 0.01	7.2 ^{g,h} ± 0.01	7.9 ^{i} ± 0.01	6.0 ± 0.07	6.2 ^{l} ± 0.02	6.3 ^{n} ± 0.01	6.6 ^{i} ± 0.06
PO23	4.9 ± 0.2	5.3 ^{b} ± 0.01	5.8 ^{b} ± 0.01	6.4 ^{c} ± 0.09	6.0 ± 0.07	6.1 ^{h,i} ± 0.01	6.2 ^{m,n} ± 0.01	6.4 ^{h,i} ± 0.02
RO1	4.9 ± 0.2	5.7 ^{c,d} ± 0.12	5.9 ^{b} ± 0.11	5.0 ^{a} ± 0.01	6.0 ± 0.07	5.4 ^{a} ± 0.01	4.4 ^{a} ± 0.10	4.2 ^{a} ± 0.01
RO2	4.9 ± 0.2	6.9 ^{i} ± 0.13	7.2 ^{g,h} ± 0.07	7.3 ^{g,h} ± 0.19	6.0 ± 0.07	5.4 ^{a} ± 0.01	4.8 ^{b} ± 0.08	4.3 ^{a} ± 0.03
RO20	4.9 ± 0.2	5.4 ^{b,c} ± 0.01	5.8 ^{b} ± 0.09	6.8 ^{d,e} ± 0.07	6.0 ± 0.07	5.7 ^{c} ± 0.02	5.4 ^{c,d} ± 0.01	5.6 ^{d,e,f} ± 0.11
RO21	4.9 ± 0.2	6.3 ^{f,g,h} ± 0.11	6.4 ^{c,d} ± 0.02	7.3 ^{f,g,h} ± 0.08	6.0 ± 0.07	5.9 ^{d} ± 0.02	5.6 ^{e} ± 0.10	4.8 ^{b} ± 0.04
RO22	4.9 ± 0.2	4.6 ^{a} ± 0.08	5.0 ^{a} ± 0.02	5.0 ^{a} ± 0.01	6.0 ± 0.07	6.0 ^{e,f} ± 0.03	5.6 ^{e} ± 0.01	4.9 ^{b} ± 0.01
RO7	4.9 ± 0.2	5.4 ^{b,c} ± 0.40	6.4 ^{c,d} ± 0.05	6.3 ^{b,c} ± 0.01	6.0 ± 0.07	6.0 ^{f,g} ± 0.01	6.0 ^{h,i} ± 0.01	5.8 ^{e,f} ± 0.15
RO8	4.9 ± 0.2	5.9 ^{d,e,f} ± 0.15	6.5 ^{c,d} ± 0.11	7.1 ^{e,f,g} ± 0.23	6.0 ± 0.07	6.0 ^{g,h} ± 0.02	6.1 ^{i,l} ± 0.01	6.6 ^{i} ± 0.21
RO9	4.9 ± 0.2	6.4 ^{g,h} ± 0.02	6.9 ^{f,g} ± 0.1	7.6 ^{h,i} ± 0.13	6.0 ± 0.07	6.0 ^{f,g} ± 0.01	5.8 ^{f,g} ± 0.06	5.5 ^{c,d,e} ± 0.23
Y2	4.9 ± 0.2	6.5 ^{h} ± 0.05	7.0 ^{f,g} ± 0.18	6.6 ^{c,d} ± 0.01	6.0 ± 0.07	5.9 ^{d,e} ± 0.01	5.4 ^{d} ± 0.04	5.3 ^{c} ± 0.18
Y20	4.9 ± 0.2	6.1 ^{e,f,g} ± 0.01	6.2 ^{c} ± 0.03	6.3 ^{b,c} ± 0.02	6.0 ± 0.07	6.1 ^{i} ± 0.01	5.7 ^{e,f} ± 0.01	4.4 ^{a} ± 0.04
Y21	4.9 ± 0.2	6.2 ^{e,f,g,h} ± 0.12	6.8 ^{e,f} ± 0.1	7.0 ^{e,f} ± 0.20	6.0 ± 0.07	6.1 ^{i} ± 0.03	6.3 ^{m,n} ± 0.03	6.1 ^{g,h} ± 0.06
Y22	4.9 ± 0.2	6.3 ^{g,h} ± 0.08	6.9 ^{f,g} ± 0.04	7.1 ^{e,f,g} ± 0.02	6.0 ± 0.07	5.9 ^{d,e} ± 0.03	5.3 ^{c} ± 0.04	4.9 ^{b} ± 0.01
Y5	4.9 ± 0.2	6.5 ^{h} ± 0.07	6.6 ^{d,e} ± 0.16	6.9 ^{d,e} ± 0.02	6.0 ± 0.07	5.9 ^{d} ± 0.04	5.5 ^{d} ± 0.04	5.3 ^{c,d} ± 0.05
Y6	4.9 ± 0.2	6.2 ^{f,g,h} ± 0.09	6.5 ^{c,d} ± 0.04	7.0 ^{e,f,g} ± 0.30	6.0 ± 0.07	5.7 ^{b} ± 0.02	5.7 ^{e,f} ± 0.01	5.8 ^{e,f} ± 0.02

For the strains incubated in squacquerone whey (Table 4), only PO23 and RO22 showed slow or no growth over time. Among the remaining ones, the highest increase in cell count (1.3 log cfu/mL) was already observed after 24 h, while it reduced to 0.5 and 0.4 log cfu/mL after 48 and 72 h, respectively. The most active yeast was RO2 (8.3 log cfu/mL), followed by PO1, PO19, PO20, PO2, RO21, RO9, Y2, Y21, Y22, Y5 and Y6, that reached a final concentration of at least 6.9 log cfu/mL. Looking at pH behavior, this had the same trend observed in caciotta whey, with a decrease of around 1.3 after 48 h. The lowest pH was measured in the sample inoculated with PO23 (pH 4.2), while the highest (5.8) was observed in the sample with RO2.

The trial was also performed on the whey mix. However, due to the initial pH (3.6) the growth of *Y. lipolytica* was tremendously reduced. In fact, a mean increase of 0.4 log cfu/mL for all the strains was observed after 72 h incubation (data not shown). Therefore, this type of whey was not taken into consideration for further trials.

Since LAB were present at the beginning of the fermentation, especially for caciotta and squacquerone whey, and pH decreased over time during 72 h incubation, their counts were estimated in the samples with the three best growing strains (PO1, PO2, and RO2). The LAB, when inoculated with PO1, PO2 and RO2, respectively, reached the following concentrations: 7.5 ± 0.15, 7.3 ± 0.01, and 7.0 ± 0.07 log cfu/mL in caciotta whey; 6.7 ± 0.10, 6.8 ± 0.21, and 7.7 ± 0.14 in ricotta whey; and 8.1 ± 0.07, 8.0 ± 0.09, and 7.0 ± 0.08 in squacquerone whey.

Table 4. Concentrations of *Y. lipolytica* in squacquerone whey and pH over time. Results are the mean of three different measurements. Different letters mean significant differences ($p < 0.05$) within a timepoint.

	Squacquerone Whey							
	Concentration (log CFU/g)				pH			
		24 h	48 h	72 h	0 h	24 h	48 h	72 h
PO1	4.9 ± 0.2	6.5 ^{h,i,l} ± 0.10	7.2 ^{i} ± 0.26	7.7 ^{h} ± 0.02	6.5 ± 0.11	6.8 ^{b} ± 0.05	5.8 ^{l} ± 0.04	5.1 ^{g,h} ± 0.21
PO10	4.9 ± 0.2	5.9 ^{e} ± 0.08	6.3 ^{d,e} ± 0.02	6.5 ^{c,d} ± 0.01	6.5 ± 0.11	6.6 ^{f,g} ± 0.01	5.4 ^{g,h,i} ± 0.01	4.9 ^{d,e,f} ± 0.12
PO19	4.9 ± 0.2	6.2 ^{f,g,h} ± 0.04	6.8 ^{f,g,h,i} ± 0.06	7.6 ^{g,h} ± 0.12	6.5 ± 0.11	6.6 ^{f,g} ± 0.02	4.9 ^{b} ± 0.09	4.4 ^{a,b} ± 0.03
PO2	4.9 ± 0.2	6.4 ^{g,h,i,l} ± 0.06	6.9 ^{g,h,i} ± 0.19	7.3 ^{f,g} ± 0.05	6.5 ± 0.11	6.6 ^{e,f,g} ± 0.02	5.2 ^{d,e,f,g} ± 0.01	4.9 ^{d,e,f} ± 0.09
PO20	4.9 ± 0.2	6.0 ^{e,f} ± 0.11	6.4 ^{e} ± 0.10	6.9 ^{e,f} ± 0.14	6.5 ± 0.11	6.5 ^{d,e,f,g} ± 0.01	5.1 ^{b,c,d,e} ± 0.01	4.8 ^{c,d} ± 0.01
PO23	4.9 ± 0.2	5.0 ^{b} ± 0.01	5.1 ^{b} ± 0.20	5.7 ^{b} ± 0.26	6.5 ± 0.11	6.4 ^{c,d} ± 0.03	4.5 ^{a} ± 0.12	4.2 ^{a} ± 0.02
RO1	4.9 ± 0.2	6.5 ^{i,l} ± 0.06	6.6 ^{e,f,g} ± 0.26	6.5 ^{c,d} ± 0.19	6.5 ± 0.11	6.0 ^{a} ± 0.01	4.5 ^{a} ± 0.21	4.4 ^{b} ± 0.01
RO2	4.9 ± 0.2	7.6 ^{m} ± 0.04	8.4 ^{l} ± 0.05	8.3 ^{i} ± 0.08	6.5 ± 0.11	6.4 ^{c,d} ± 0.11	6.5 ^{m} ± 0.10	5.8 ^{i} ± 0.03
RO20	4.9 ± 0.2	5.1 ^{b,c} ± 0.01	5.7 ^{c} ± 0.09	6.2 ^{c} ± 0.29	6.5 ± 0.11	6.4 ^{c} ± 0.01	5.3 ^{e,f,g,h} ± 0.03	5.1 ^{f,g,h} ± 0.03
RO21	4.9 ± 0.2	6.2 ^{f,g} ± 0.08	6.4 ^{d,e} ± 0.04	7.2 ^{f,g} ± 0.11	6.5 ± 0.11	6.5 ^{c,d,e,f} ± 0.09	4.9 ^{b} ± 0.05	4.9 ^{d,e} ± 0.01
RO22	4.9 ± 0.2	4.1 ^{a} ± 0.02	4.4 ^{a} ± 0.07	5.0 ^{a} ± 0.01	6.5 ± 0.11	6.6 ^{e,f,g} ± 0.01	5.6 ^{i,l} ± 0.01	5.2 ^{h} ± 0.01
RO7	4.9 ± 0.2	6.0 ^{e,f} ± 0.21	6.4 ^{d,e} ± 0.05	6.3 ^{c} ± 0.01	6.5 ± 0.11	6.7 ^{b,g} ± 0.04	5.5 ^{h,i} ± 0.04	5.1 ^{e,f,g,h} ± 0.05
RO8	4.9 ± 0.2	6.1 ^{e,f} ± 0.19	5.9 ^{c,d} ± 0.17	6.7 ^{d,e} ± 0.08	6.5 ± 0.11	6.6 ^{e,f,g} ± 0.05	5.3 ^{f,g,h,i} ± 0.01	4.8 ^{c,d} ± 0.01
RO9	4.9 ± 0.2	6.4 ^{h,i,l} ± 0.02	6.9 ^{f,g,h,i} ± 0.05	7.0 ^{e,f} ± 0.09	6.5 ± 0.11	6.6 ^{f,g} ± 0.12	5.3 ^{e,f,g} ± 0.01	4.9 ^{d,e,f} ± 0.04
Y2	4.9 ± 0.2	6.6 ^{l} ± 0.01	7.1 ^{h,i} ± 0.01	7.2 ^{f} ± 0.16	6.5 ± 0.11	6.5 ^{c,d,e,f} ± 0.03	4.9 ^{b} ± 0.07	4.6 ^{c} ± 0.01
Y20	4.9 ± 0.2	5.5 ^{d} ± 0.12	5.7 ^{c} ± 0.20	6.5 ^{c,d} ± 0.10	6.5 ± 0.11	6.5 ^{c,d,e,f,g} ± 0.01	5.1 ^{b,c,d,e,f} ± 0.01	4.8 ^{c,d} ± 0.05
Y21	4.9 ± 0.2	5.3 ^{c,d} ± 0.11	6.5 ^{e,f} ± 0.02	7.0 ^{e,f} ± 0.04	6.5 ± 0.11	6.6 ^{d,e,f,g} ± 0.01	5.1 ^{c,d,e,f} ± 0.01	5.0 ^{d,e,f,g} ± 0.01
Y22	4.9 ± 0.2	6.1 ^{e,f} ± 0.01	6.7 ^{e,f,g} ± 0.15	7.2 ^{f} ± 0.01	6.5 ± 0.11	6.5 ^{c,d,e} ± 0.05	4.9 ^{b,c} ± 0.09	4.8 ^{c,d} ± 0.01
Y5	4.9 ± 0.2	6.1 ^{e,f} ± 0.02	6.8 ^{f,g,h,i} ± 0.24	6.9 ^{e,f} ± 0.03	6.5 ± 0.11	6.5 ^{d,e,f,g} ± 0.04	5.3 ^{e,f,g} ± 0.01	4.9 ^{d} ± 0.03
Y6	4.9 ± 0.2	6.4 ^{g,h,i} ± 0.01	6.8 ^{e,f,g,h} ± 0.15	7.1 ^{f} ± 0.01	6.5 ± 0.11	6.6 ^{d,e,f,g} ± 0.03	5.0 ^{b,c,d} ± 0.21	4.8 ^{c,d} ± 0.01

3.3. Volatile Molecule Producation in Different Types of Whey by the Three Best Growing Strains

The samples obtained using the best growing strains (PO1, PO2, and RO2) in the three different types of whey were characterized for the volatile molecules produced.

In caciotta whey, 68 volatile molecules were identified, mainly belonging to esters, alcohols, and ketones. To better highlight the effects of the different strains, a Principal Component Analysis (PCA) was performed with volatilome data.

The projection of the samples is reported in Figure 4a, where PC1 and PC2 could explain 56.2 and 27.4% of the total variance among the samples, respectively. All the samples containing *Y. lipolytica* projected far from the control, that was incubated for 72 h. At the same time, each strain imparted a different profile since they were distributed in three different quarters of the PCA figure. The control sample was characterized mainly by the presence of alcohols (i.e., 2,4−dimethyl−3−pentanol, 2−methyl−1−propanol, 2−methyl−1−butanol), acids (i.e., acetic acid, propanoic acid 2 methyl), esters (ethyl acetate, propanoic acid 2−methylpropyl ester) and alkanes (3,3−dimethyl−hexane, 3,7−dimethyl−undecane). On the contrary, RO2 was characterized by the presence of 5−methyl−3−hexanone, 2−pentyl−thiophene, and pentanoic acid ethyl ester, while PO1 was characterized by 2−butanone, diacetyl, and methyl isobutyl ketone. The PO2 was characterized by a higher amount of ethanol, 2−nonanone, 2−hexanol, and different ester compounds (butanoic acid propyl ester, hexanoic acid ethyl ester, pentanoic acid ethyl ester, butanoic acid butyl ester, hexanoic acid 2−methylproyl ester, caprylic acid isobutyl ester, decanoic acid ethyl ester) (Figure 4b).

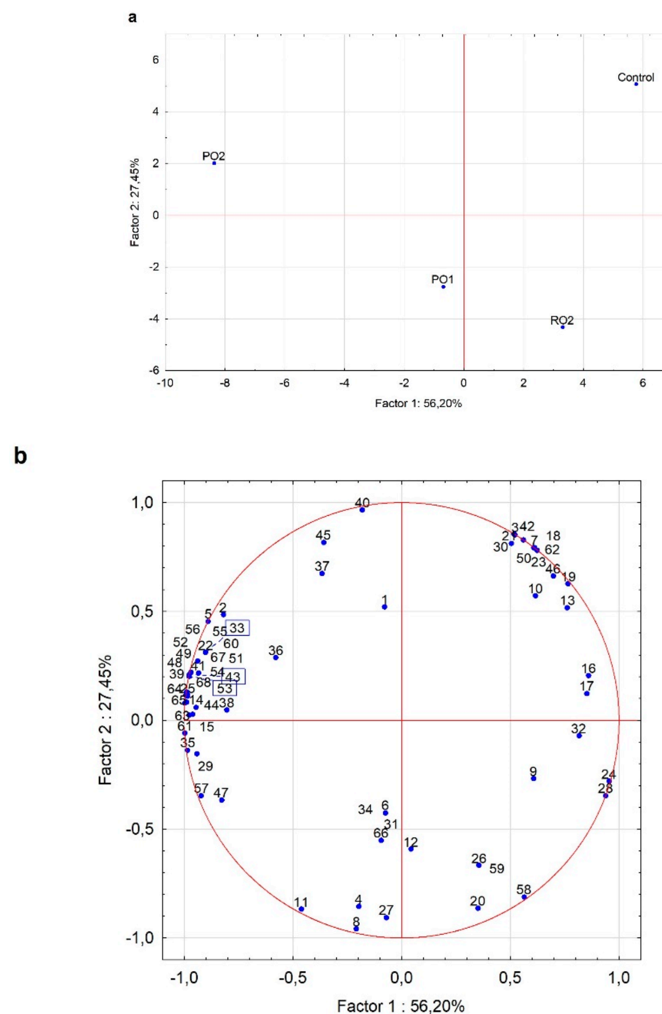

Figure 4. Projection on the factor plane (1 × 2) of caciotta whey inoculated with three different strains of *Y. lipolytica* (RO2, PO1, PO2) and incubated for 72 h. The control was represented by caciotta whey without *Y. lipolytica*. (**a**) Treatments; (**b**) Variables. 1: 4−methyl−octane; 2: 2,4−dimethyl−1−heptene; 3: ethyl acetate; 4: 2−butanone; 5: ethanol; 6: 2,2,4,6,6−pentamethyl−heptane; 7: propionic acid, ethyl ester; 8: diacetyl; 9: 2−pentanone; 10: 3,3−dimethyl−hexane; 11: methyl isobutyl ketone; 12: dodecane; 13: 3,7−dimethyl−undecane; 14: 1−propanol; 15: butanoic acid, ethyl ester; 16: 2,3−dimethyl−decane; 17: 3,7−dimethyl−decane; 18: acetic acid, butyl ester; 19: propanoic acid, 2−methylpropyl ester; 20: 5−methyl−3−hexanone; 21: 2−methyl−1−propanol; 22: butanoic acid, propyl ester; 23: 3−methyl−acetate−1−butanol; 24: 4−methyl−2−hexanone; 25: pentanoic acid, ethyl ester; 26: 2,4,6−trimethyl−octane; 27: 4−methyl−3−penten−2−one; 28: 2,6−dimethyl−4−heptanone; 29: 2−heptanone; 30: 2−methyl−1−butanol; 31: 2,3,6−trimethyl−decane; 32: 2−hexanol; 33: butanoic acid, butyl ester; 34: 5−methyl−hexanol; 35: hexanoic acid, ethyl ester; 36: pentanoic acid, 1−methylpropyl ester; 37: acetoin; 38: hexanoic acid, propyl ester; 39: heptanoic acid, ethyl ester; 40: 1−hexanol; 41: hexanoic acid, 2−methylproyl ester; 42: 2,4−dimethyl−3−pentanol; 43: 2−nonanone; 44: benzene−1,3 bis−1,1−dimethylethyl; 45: 1−heptanol; 46: acetic acid; 47: 2−ethyl−1−hexanol; 48: nonanoic acid, ethyl ester; 49: caprylic acid, isobutyl ester; 50: propanoic acid, 2 methyl; 51: hexadecane; 52: butyl caprylate; 53: butanoic acid; 54: decanoic acid, ethyl ester; 55: 2−octanol; 56: octanoic acid, 3−methylbutyl ester; 57: benzoic acid, ethyl ester; 58: 2−pentyl−thiophene; 59: pentanoic acid; 60: capric acid, isobutyl ester; 61: hexanoic acid; 62: acetic acid, 2 phenylethyl ester; 63: octanoic acid; 64: nonanoic acid; 65: decanoic acid; 66: phenol 2,4−bis 1,1−dimethylethyl; 67: 9−decenoic acid; 68: dodecanoic acid.

In ricotta whey, 26 volatile molecules were identified, mainly belonging to ketones and alcohols. A PCA was performed to better highlight the effects of the different strains on the volatilome profile. The projection of the samples is reported in Figure 5a, where PC1 and PC2 could explain the 48.9 and 39.3%, respectively, of the total variance among the samples. All the samples containing *Y. lipolytica* projected far from the control. At the same time, each strain conferred a different profile to the product. PO1 and RO3 clustered close and on the opposite side from the control. The control sample was mainly characterized by the presence of 2−ethyl−1−hexanol and ketones (2−pentanone, 5−methyl−3−hexanone and 2−heptanone). On the contrary, PO1 and RO2 were characterized by 4−methyl−3−penten−2−one, 2,6−dimethyl−4−heptanone and methyl isobutyl ketone. Eventually, PO2 was characterized by a higher amount of phenylethanol, short and medium chain fatty acids, and acetoin (Figure 5b).

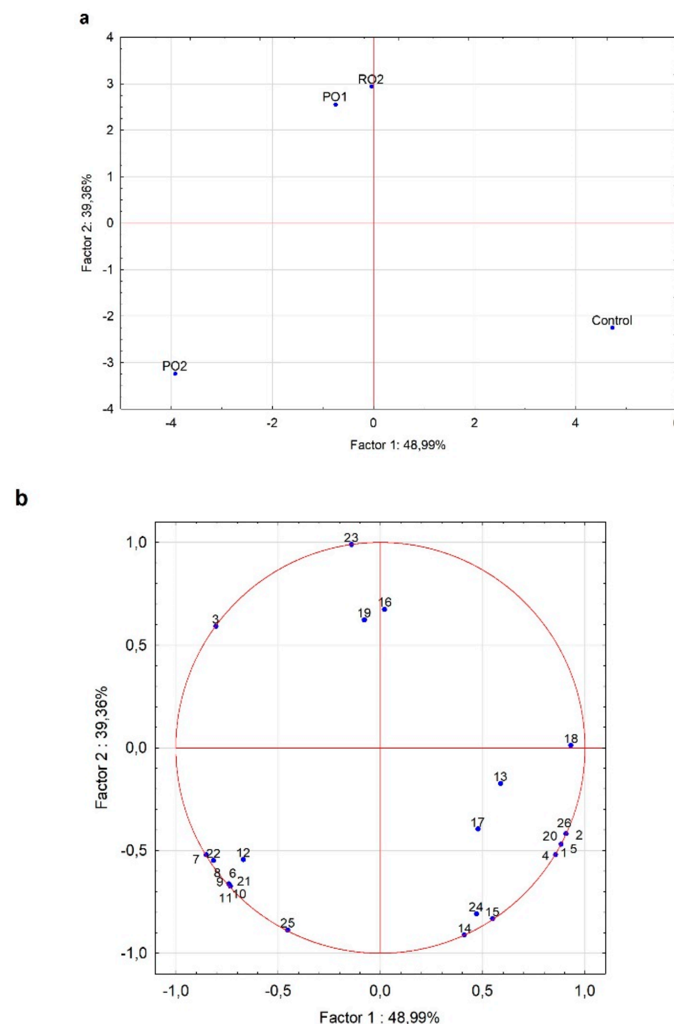

Figure 5. Projection on the factor plane (1 × 2) of ricotta whey inoculated with three different strains of *Y. lipolytica* (RO2, PO1, PO2) and incubated for 72 h. The control was represented by caciotta whey without *Y. lipolytica*. (**a**) Treatments; (**b**) Variables. 1: hexanoic acid, ethyl ester; 2: ethyl acetate; 3: 2−hexanol; 4: 2−ethyl−1−hexanol; 5: 2−methylthio−ethanol; 6: phenylethanol; 7: acetic acid; 8: butanoic acid; 9: hexanoic acid; 10: octanoic acid; 11: decanoic acid; 12: acetone; 13: 2−butanone; 14: 3−methyl−butanal; 15: 2−pentanone; 16: methyl isobutyl ketone; 17: 5−methyl−3−hexanone; 18: 4−methyl−2−hexanone; 19: 2,6−dimethyl−4−heptanone; 20: 2−heptanone; 21: acetoin; 22: phenylacetaldehyde; 23: 4−methyl−3−penten−2−one; 24: heptadecane; 25: hexadecane; 26: benzene, 1,3−bis(1,1−dimethylethyl).

In squacquerone whey, 50 volatile molecules were identified, and they mainly belonged to ketones, acids and alcohols. On the PCA performed with the volatilome data, PC1 and PC2 could explain, respectively, 39.4 and 35.6% of the total variance among the samples (Figure 6a).

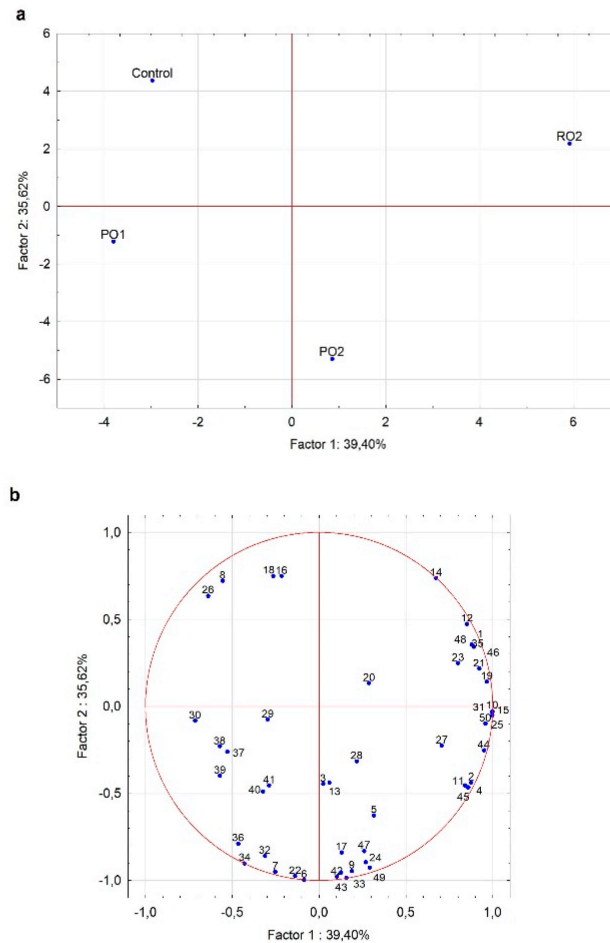

Figure 6. Projection on the factor plane (1 × 2) of squacquerone whey inoculated with three different strains of *Y. lipolytica* (RO2, PO1, PO2) and incubated for 72 h. The control was represented by caciotta whey without *Y. lipolytica*. (**a**) Treatments; (**b**) Variables. 1: butanoic acid, ethyl ester; 2: hexanoic acid, ethyl ester; 3: oxalic acid, 6−ethyloct−3yl−ethyl ester; 4: propanoic acid, 2−methyl ester; 5: pentanoic acid, 1,1−dimethylethyl ester; 6: acetone; 7: 2−butanone; 8: 2,3−butanedione; 9: 2−pentanone; 10: methyl isobutyl ketone; 11: 5−methyl−3−hexanone; 12: 3,4−dimethyl−2−pentanone; 13: 4−methyl−2−hexanone; 14: 2,6−dimethyl−4−heptanone; 15: 2−heptanone; 16: acetoin; 17: acetoin; 18: 2−methyl−3−decen−5−one; 19: ethanol; 20: 2−methyl−1−propanol; 21: 3−methyl−1−butanol; 22: 2−hexanol; 23: 3−heptanol; 24: 2−ethyl−1−hexanol; 25: phenylethanol; 26: acetic acid; 27: butanoic acid; 28: butanoic acid, 2−methyl; 29: pentanoic acid; 30: hexanoic acid; 31: hexanoic acid, 4−methyl; 32: octanoic acid; 33:nonanoic acid; 34: decanoic acid; 35: undecanoic acid; 36: dodecanoic acid; 37: 4−methyl−octane; 38: 2,4−dimethyl−1−heptene; 39: 2,2,4,6,6−pentamethyl−heptane; 40: 2,4−dimethyl−decane; 41: 3,7−dimethyl−undecane; 42: 3,7−dimethyl−decane; 43: 3,6−dimethyl−decane; 44: heptadecane; 45: tridecane, 6−methyl; 46: pentadecane; 47: hexadecane; 48: gamma−decanolactone; 49: benzene, 1,3−bis(1,1−dimethylethyl); 50: phenol, 2,4−bis(1,1 dimethylethyl).

All the samples containing *Y. lipolytica* projected far from the control in three different quarters. The control sample was characterized mainly by ketones (mainly acetoin, 2−methyl−3−Decen−5−one, diacetyl) and acids (e.g., acetic acid), while PO1 was characterized by alkanes (4−methyl−octane, 2,4−dimethyl−1−heptene and 2,2,4,6,6−pentamethyl−

heptane, 2,4−dimethyl−decane) and acids (pentanoic acid, hexanoic acid, octanoic acid). The RO2 was characterized by the presence of undecanoic acid, 3,4−dimethyl−2−pentanone, 2,6−dimethyl−4−heptanone, butanoic acid ethyl ester and gamma−decalactone, among others. Eventually, PO2 was characterized by higher amounts of esters (propanoic acid 2−methyl ester, pentanoic acid 1,1−dimethylethyl ester, hexanoic acid ethyl ester), 5−methyl−3−hexanone, 2−ethyl−1−hexanol, and benzene, 1,3−bis(1,1−dimethylethyl) (Figure 6b).

4. Discussion

The use of agri-food waste and by-products as substrates to grow microorganisms is acquiring more and more interest. In our work, the use of cheese whey as a substrate for *Y. lipolytica* growth was evaluated. According to the literature, most of the works that applied *Y. lipolytica* for food waste and by-product valorization mainly focused on the production of value added compounds, such as citric acid, single cell oils etc. [1]. The production of enriched microbial cultures was always a secondary aspect that came from the first process. Moreover, the uses of these biomasses as food grade starters, adjuncts cultures or food ingredients were not always considered. Cheese whey has been used as a substrate for lactic acid bacteria or other filamentous fungi to produce biomasses or added value compounds. For example, Braz et al. [33] tested raw cheese whey as a culture medium for *Mucor circinelloides* URM 4182 to produce a biomass containing lipids and lipase. On the other hand, Costa et al. [34] produced lactic acid by fermenting ricotta cheese whey with *Lacticaseibacillus casei* DSM 20011, while Brizuela et al. [35] used whey permeate as a substrate for the production of freeze-dried *Lactiplantibacillus plantarum* to be used as a malolactic starter culture.

In this work, we first characterized 20 strains of *Y. lipolytica* isolated from different environments for their food-related enzymatic properties (hydrolysis of dairy proteins and fats), with the aim of producing co-starter and adjunct cultures for the dairy industry. The proteolytic capacity was fundamental during the ripening of several cheeses, and it strongly affected flavor and texture development [36–39]. In fact, the conversion of caseins into low MW peptides and free amino acids had a direct impact on cheese flavor or could generate precursors of flavor compounds [40,41]. All the strains showed this capability with origin-related and strain-specific patterns. In fact, the PO strains isolated from Po River showed the strongest proteolysis of both α- and β-caseins, followed by RO and Y strains. Some of these strains (4 PO and 4 RO strains) had already been tested by Vannini et al. [42]. Even in that case, PO strains caused the strongest protein hydrolysis. However, no complete casein proteolysis was observed since the incubation was performed only for 24 h. In our study, the incubation was stopped after 72 h and, therefore, a complete consumption of the proteins was observed on the SDS–page. The proteolytic potential of *Y. lipolytica* is well known and it has been reported in several works [28,43–45]. This potential is based on the activity of two extra-cellular proteases, one alkaline serine protease and one acid aspartic protease [1].

On the other hand, lipolysis represents the first feature characterizing *Y. lipolytica* [26,27,30,43,46,47]. Several studies revealed 25 putative lipases and, among them, some are extracellular (Lip2), while others are intracellular (Lip1, Lip3 and Lip6) or cell-bounded enzymes (Lip7, Lip8) [48–50]. These enzymes show a substrate specificity ranging from medium- to long-chain fatty acids. The released fatty acids can be further transformed into desirable or undesirable volatile or non-volatile compounds with characteristic aromas [27,51]. The selection of strains of *Y. lipolytica* to be used in the dairy sector should be based on a deeper knowledge of their activities. In our work, strain-specific FFA patterns were observed. These profiles and yeast behaviors were in line with what was already described by Guerzoni et al. [30], where some of these strains were tested. Furthermore, in our work, oleic acid (C18:1) was the main fatty acid released upon incubation with the tested strains. It accounted for 69.8 and 66.5% of the total FFAs with RO21 and PO20, respectively, while it did not exceed 16% in samples containing PO10, RO8 and Y22. The large variability observed within these strains of *Y. lipolytica* could have an indirect effect on

the subsequent transformation of the fatty acids into aroma compounds [27]. In the second part of the work, different types of whey were assessed as possible growth substrates for *Y. lipolytica*. The pH represented one of the critical variables for yeast development. In fact, the low pH of mixed whey (3.6) slowed down the cell growth of all the strains tested, since only an increase of about 0.4 log cfu/mL was observed after 72 h. On the other hand, the three other types of whey, having pH between 6.0 and 6.3, allowed the growth of almost all the strains, except for RO22, followed by RO20 and PO23, which did not grow on caciotta and squacquerone whey, respectively. On the other hand, strains PO1, PO2, RO2, RO9, Y5 and Y6 grew well in all the substrates, with PO1 and PO2 being the best growers on caciotta and ricotta whey and RO2 on squacquerone whey. The capability of *Y. lipolytica* to grow on cheese whey is quite controversial. In fact, all the data available in literature describe this yeast as a lactose-negative yeast [22]. However, Taskin et al. [23] isolated a strain (B9) that was able to assimilate lactose as a carbon source. In fact, it is known that carbon source assimilation is a highly variable phenotype that can change among different strains of the same microbial species [52]. Most of the publications related to *Y. lipolytica* did not use whey as such but in a deproteinized form or supplemented with fructose [23–25]. In fact, their main objectives were to obtain compounds, such as single cell oils and citric acid [1], that require a high C:N ratio in the medium. If lactose is not assimilated, this ratio is low, and supplementation or removal of constituents is required. In our work we could not infer that all the strains developing in cheese whey were lactose positive. Actually, *Y. lipolytica* was probably forced to use the available proteins and fats, which were present in all the types of whey, in not negligible amounts (6.0–10.7 and 2.9–4.7 g/L, respectively). This step might promote a kind of pre-adaptation (activation of the metabolisms for protein and lipid consumption) of the strains that could be more effective once added to the dairy product. The pre-adaptation of strains into substrates that could resample the final product where they were added was crucial and well demonstrated [53–55]. In regard to what concerns the unused and remaining lactose, it could be used to extend yeast storage, since it is a key component of cryoprotectants and lyoprotectans [56–59].

The pH reduction observed in almost all the samples of caciotta, squacquerone and, partially, ricotta whey, incubated with *Y. lipolytica*, was caused by acid production. In our work, whey was collected in a sterile way and immediately inoculated with *Y. lipolytica*. However, due to the presence of starter cultures deriving from cheesemaking, spontaneous whey fermentation took place. This process mostly depended on LAB, which convert lactose into lactic acid or proteins into functional peptides or amino acids [60]. All these compounds could then be used by *Y. lipolytica* as substrates [61]. According to Mansour et al. [61], if sugars are not available, *Y. lipolytica* would use, first, amino acids as a main energy source, while lactate would be consumed following amino acid depletion. Amino acid degradation is usually accompanied by ammonia production, corresponding to a dramatic increase in pH. This would explain the pH stability or increase observed in samples of ricotta whey. In fact, ricotta is made without LAB culture addition and, therefore, only native LAB, coming from the environment or that survived the cheesemaking process (which include a thermal treatment), may have grown. The initial concentration was indeed below the limit of quantification (<1 log cfu/mL). Taking all these information together, it is reasonable to think that, in our work, a co-culture of *Y. lipolytica* strains and LAB had occurred, meaning that some strains, especially the best growers, might have taken advantage from bacterial growth, or, at least, were not affected by it. A combination of *Y. lipolytica* and LAB had already been reported in literature [62]. Moreover, *Y. lipolytica* can persist in microbial consortia without being affected by other microorganisms [63]. This is relevant if *Y. lipolytica* is used as a possible cheese culture adjunct since it should not outcompete or inhibit the starter cultures during cheesemaking. A solution to avoid the presence of the microbial background could be the use of thermal treatments on cheese whey before *Y. lipolytica* inoculation. However, this step would make the process less sustainable and more expensive, particularly if performed by cheesemakers that could potentially use the

enriched-cell product directly in their productions, pursuing and addressing the goals of circular economy and sustainability.

The last aspect that was considered for strain characterization, was the production of volatile compounds. This is also important if the whole whey enriched in yeast cells would be directly used as a starter or adjunct culture. Volatile molecules produced in the different types of cheese whey were determined for the three best growers, namely, PO1, PO2 and RO2. Usually cheese yeasts can contribute to producing compounds, such as ethanol, carboxylic acids, esters, secondary alcohols, phenyl compounds, NH_3, lactones, sulfur compounds, and methyl ketones [2]. In cheese, aroma development depends on the production of volatile compounds, such as sulfur compounds (i.e., methanethiol, dimethyl sulfide or dimethyl disulfide) [64–66]. Despite the LAB background, the yeasts inoculated in cheese whey produced characteristic compounds. Although there were no specific molecules produced by each strain in the three different types of whey, samples containing PO2 were characterized by a higher abundance of esters (i.e., hexanoic acid ethyl ester, butanoic acid propyl ester, hexanoic acid 2-methylproyl ester, caprylic acid isobutyl ester, decanoic acid ethyl ester), alcohols (2-hexanol, phenylethanol, and 2-ethyl-1-hexanol) and short and medium chain fatty acids. RO2 samples were characterized mainly by ketones (such as 5-methyl-3-hexanone, 4-methyl-3-penten-2-one, 2,6-dimethyl-4-heptanone, and 3,4-dimethyl-2-pentanone), and esters (pentanoic acid ethyl ester, butanoic acid ethyl ester), while PO1 samples were characterized by acids (pentanoic acid, hexanoic acid, octanoic acid), and ketones (2-butanone, diacetyl, 4-methyl-3-penten-2-one, 2,6-dimethyl-4-heptanone). In our case, no sulfur compounds were observed, either because the incubation time (72 h) was not the same as that applied for cheese ripening (usually ranging from weeks to years [67]), or because these compounds have very low detection limits and might require the use of specific analytical methodologies [68]. Most of the detected compounds, especially esters, short and medium chain fatty acids, and ketones are characterized by fruity or buttery/creamy aromas, and they are compatible with cheese flavors and cheese-making. They could be generated by precursors deriving from hydrolyzed lipids and proteins [69]. In our work, their productions were the results of both *Y. lipolytica* and LAB strains present in the different types of whey. However, the presence of the yeasts had a strong impact, since these samples clustered separately from the respective controls. Looking specifically at the three types of whey, caciotta whey was the substrate that allowed the production of the highest number of volatile molecules (68 compared to 26 and 50 detected in ricotta and squacquerone whey, respectively). This might be due to the lipid and protein contents (around 15.4, 9.4 and 12.8 g/L in caciotta, ricotta and squacquerone whey, respectively) available for *Y. lipolytica*.

5. Conclusions

Valorization of cheese whey represents an interesting challenge for the dairy industry and several biotechnological options have been proposed. The use of dairy waste as a substrate to obtain *Y. lipolytica* cultures, to be used as starter or food adjunct in cheese, may represent a sustainable advantage, from environmental and economical points of view, to solve the issues related to waste disposal. Although further trials are required to optimize the processes and assess the feasibility at pilot scale, *Y. lipolytica* (particularly strain PO1, PO2 and RO2) grown in untreated whey may open a novel field of research. Moreover, the effect of these yeasts during cheese ripening needs to be evaluated.

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