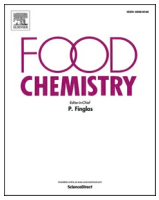
Food Chemistry 470 (2025) 142657

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Biological activities, biosynthetic capacity and metabolic interactions of lactic acid bacteria and yeast strains from traditional home-made kefir

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ARTICLE INFO

Keywords:
Metabolomics
Probiotics
Postbiotics
Fermented foods
Pichia kudriavzevii

ABSTRACT

Given the widespread industrial and domestic use of probiotic blends based on combinations of lactic acid bacteria (LAB) and yeasts to produce fermented foods or beverages that are supposed to provide health benefits, this study aimed to generate knowledge and concepts on biologically relevant activities, metabolism and metabolic interactions in yeast/LAB communities. For this, the postbiotic capabilities of three probiotic candidates, including two lactic acid bacteria (i.e., *Lactococcus lactis* subsp. *hordniae* and *Lactococcus lactis* subsp. *lactis*) and the yeast *Pichia kudriavzevii*, isolated from a traditional home-made kefir, were explored combining an assortment of bioassays with a GC–MS footprint metabolomic strategy. Cell-free supernatants from cultures showed antimicrobial/antioxidant activity and inhibited biofilm formation by *Salmonella* sp. Several bioactive secondary metabolites (including tyrosol, phenylethyl alcohol, 2,3-butanediol, erythritol, tryptophol, putrescine, cadaverine, 3-phenyllactate, 2-hydroxyisocaproate) were detected which may contribute to the odor and flavour of the fermented products and their effects on human body.

1. Introduction

The human microbiota is engaged in a mutual relationship with the host and this harmonious co-existence is important for the maintenance of health and wellbeing. The microbiota confers numerous benefits on the host, including the production of nutrients and resistance to pathogen invasion. Consequently, alterations in the balance between beneficial symbionts and commensals represent possible risk factors that could lead to disorders and diseases (Gebrayel et al., 2022; Sánchez et al., 2017).

The modulation of the human microbiota, especially the gut microbiota, is particularly regarded to maintain a favorable host-microbial association (Gebrayel et al., 2022; Sánchez et al., 2017; Schmidt et al., 2018). In fact, live microbial supplements are widely used for the promotion and improvement of health in humans. The utilization of live microorganisms as food supplement has a long history, but the addition of scientific knowledge on their traditional uses has allowed them to gain more and more attention.

According to the World Health Organization (FAO/WHO Joint Expert Consultation Report, 2001), probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host and, currently, they are among the most frequently used nutritional supplements (Reid, 2016; Sanders et al., 2018).

Fermented foods have a potential for the delivery of probiotic microorganisms into the human intestine (Marco et al., 2017). For instance, kefir has long been used due to its probiotic characteristics that have been associated with an ample variety of health-promoting benefits

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https://doi.org/10.1016/j.foodchem.2024.142657
Received 9 October 2024; Received in revised form 3 December 2024; Accepted 23 December 2024
Available online 31 December 2024

(Barros et al., 2021; Rosa et al., 2017). Kefir is traditionally prepared by the fermentation of milk with kefir grains, and it contains symbiotic mixtures of lactic acid bacteria (LAB) and yeasts that produce several useful bioactive compounds (Chaudhary et al., 2022). Furthermore, the essential functions of probiotic microorganisms could be also associated to the biodetoxification of toxic chemicals and to the reduction of harmful substances (Pop et al., 2022; Salminen et al., 2010).

It was hypothesized that the health benefits of probiotics are strictly related to their microbial composition. Multi-probiotic preparations are commonly available and although some of them have not shown superior benefits (Chapman et al., 2013; Tejero-Sariñena et al., 2013), several studies demonstrate their greater efficacy compared to single strains preparations (Chapman et al., 2011). In fact, bacteria and yeasts may be used as probiotics either in the form of a single strain or combination of microorganisms acting synergistically to provide health-promoting effects and support immune system (Chapman et al., 2011; Collado et al., 2007; Salminen et al., 2010).

Homemade fermented foods could be very rich sources of microorganisms with probiotic properties because the microbial diversity is enhanced by the local traditional fermentation processes and environmental conditions (Kariyawasam et al., 2021; Rahmani et al., 2022). Hence, several efforts are presently made to isolate novel strains and characterize them for their application in functional foods.

Viability is an intrinsic attribute of probiotics and is considered a necessary ingredient for them to produce health benefits (Lahtinen, 2012); however, secreted probiotic-derived effector molecules that recently have been identified as “postbiotic” mediators could be an attractive alternative. More precisely, postbiotics are defined as preparations of inanimate microorganisms and/or their components that confer a health benefit on the host (Salminen et al., 2021). In other words, postbiotics are mixtures of microbial derived substances which mimic the beneficial properties of probiotics while avoiding the administration of live microorganisms (Aguilar-Toalá et al., 2018). Moreover, postbiotics possess additional favorable characteristics, including a clear chemical structure, safety dose parameters, long shelf life, which make the postbiotic administration a promising treatment for a variety of diseases (Rafique et al., 2023). Postbiotics can contain a panoply of bioactive compounds which are microbial metabolites that possess biological activities in humans when consumed. Healthy postbiotics include nutrients such as amino acids, vitamins, bacteriocins, antibiotics, phenolic compounds, organic acids, pigments, alkaloids which possess various antioxidant, antimicrobial and anti-inflammatory activities and help control acute and chronic diseases in humans, including cardiovascular diseases, allergies, diabetes and cancer (Prajapati et al., 2023).

Recently, our research group conducted a set of in vitro and in vivo tests on microorganisms isolated from traditional homemade kefir. This panel data set allowed us to find three potential probiotic candidates (i.e., *Pichia kudriavzevii* Y1, *Lactococcus lactis* subsp. *hordniae* LAB1 and *Lactococcus lactis* subsp. *lactis* LAB2) with desirable characteristics to be used as starter cultures to produce probiotic-based food products (Maione et al., 2024). Lactic acid bacteria (LAB) are the essential family of fermenting bacteria that produce various bioactive metabolites, including lactic acid and 3-phenyllactic acid, B vitamins, bacteriocins, γ-aminobutyric acid, ornithine, mannitol, exopolysaccharides, 2-hydroxyisocaproic acid (Lee et al., 2021; Mora-Villalobos et al., 2020). *Pichia kudriavzevii*, a yeast not considered a conventional probiotic, is ubiquitous in natural environments and is found in traditionally fermented foods and beverages in every continent of the world. This yeast has recently attracted attention for its inherent tolerance to hyperosmotic stress and pH, which makes it very suitable as a partner for LAB in food and beverage fermentation and is considered an emerging probiotic that can improve the quality of fermented products by providing aroma compounds and other metabolites (e.g., 2-phenylethanol and organic acids) beneficial to human health (Chu et al., 2023; Lata et al., 2022). Furthermore, due to its ability to control the growth of several common fungal plant pathogens, *Pichia kudriavzevii* has been suggested as a biocontrol agent in sustainable agriculture (Elkhairy et al., 2023).

The promising results obtained in our previous study (Maione et al., 2024) induced us to characterize postbiotic capabilities of these potential probiotic microorganisms. Within this framework, single and co-cultures of *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1 and L. *lactis* subsp. *lactis* LAB2 were prepared in culture broth and their cell-free supernatants (CFSs) were tested in vitro for antimicrobial, antibiofilm, antioxidant, cytotoxic and antiproliferative activities. After that, analytical data on these CFSs were collected by using a GC–MS metabolomic footprint strategy to delineate the ability of these microorganisms to synthesize bioactive compounds in single and co-cultures.

Overall, in this work we offer new insights into the postbiotic capabilities and interspecies interactions of three potential probiotic microorganisms and outline a workflow for evaluating metabolic interactions in microbial co-cultures that may be useful for the development of functional foods and dietary supplements.

2. Materials and methods

2.1. Biological Essays

2.1.1. Microbial Strains, Cultural Conditions and Growth

P. kudriavzevii Y1, *L. lactis* subsp. *hordniae* LAB1 and *L. lactis* subsp. *lactis* LAB2 isolated from a homemade kefir (Maione et al., 2024) were used in this study.

The panel of enteric bacteria used to evaluate antimicrobial activities included four *Salmonella* spp. (S1, S2, S3, S4) and one *Escherichia coli* isolated from foods; and *E. coli* ATCC 25922 and *Listeria monocytogenes* ATCC 7644, belonging to our collection. These enteric bacteria were maintained on Tryptic Soy Agar (TSA) and grown before each experiment in Tryptic Soy Broth (TSB) (VWR Chemicals, Radnor, Pennsylvania, United States).

Individual cultures and co-cultures of the three isolated strains (i.e., *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1 *and L. lactis* subsp. *Lactis* LAB2) were grown in MRS (De Man, Rogosa, and Sharpe) broth (MRS, Oxoid Ltd., Basingstoke, UK).

A pre-inoculum of LAB1 and LAB2 was prepared in MRS broth using single colonies, while the pre-inoculum of Y1 was prepared in yeast extract peptone dextrose (YPD, Oxoid Ltd.) also using single colonies. The cells were cultivated overnight at 37 °C with agitation at 180 rpm to ensure optimal aeration and growth.

On the next day, these cells were used to inoculate fresh MRS broth at ~1×10^3 cells mL^{-1} for each microorganism as required for individual cultures and co-cultures.

Cultures were incubated at 37 °C in aerobic atmosphere under shaking at 90 rpm for 48 h and growth of the three species was followed by testing the increase in cellular viability based on the number of colonies forming units (CFUs). For this, aliquots of individual cultures and co-cultures were taken, serially diluted, and plated on MRS with AMPH B or Rose Bengal Agar (RB, Sigma-Aldrich, St. Louis, MO, USA), supplemented with chloramphenicol, to distinguish LAB and Y1, respectively, and incubated at 37 °C for 24/48 h. However, in the case of the co-culture, which was prepared by inoculation of the three probiotic isolates (1:1:1 ratio), LAB species cannot be individually quantified due to their morphological similarity and, for this reason, in the co-culture the total biomass of LAB1 and LAB2 was evaluated.

As reported in detail in Table S1, after 24 h, single cultures reached approximately 3×10^8 CFU mL^{-1} while in the co-culture the growth was slower and reached totally about 8×10^7 CFU mL^{-1}.

2.1.2. Preparation of Cell-Free Supernatant (CFS)

Cell-Free Supernatants (CFSs) from individual cultures and co-cultures of the microorganisms were collected by centrifugation for 30 min at 400 rpm and 4 °C and subsequent filtration through 0.22 μm cellulose membrane filters (Corning, New York, NY, USA) and used for

GC–MS analysis as described later and for biological assays. For biological assays, CFSs were lyophilized using the HETO PowerDry PL6000, Thermo Fisher Scientific (Waltham, MA, USA). For subsequent experiments, each lyophilized CFS was reconstituted by dissolving in 2 % DMSO to obtain stock solutions of 500 mg mL^{-1} final concentration of lyophilized cell-free supernatant.

2.1.3. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of CFSs from cultures and co-cultures were evaluated by serially diluting 500 mg mL^{-1} stock solutions of lyophilized CFSs from 250 mg mL^{-1} to 10 mg mL^{-1}. The diluted samples (100 μL) were then transferred to microplate wells with triplicates and ten microliters of each pathogen culture was inoculated into each well. The experimental plates were incubated for 24 h at 37 °C. MIC was evaluated measuring absorbance at 600 nm.

2.1.4. Biofilm formation and eradication assays and effect of CFSs

For biofilm formation and eradication assays, the *Salmonella* sp. S1 biofilm cultures were grown in 96-well flat-bottomed polystyrene microtiter plates (Eppendorf, Hamburg, Germany) starting from an inoculum concentration in each well of 1.0×10^6 CFU mL^{-1}.

In order to detect the ability of *Salmonella* sp. S1 to form biofilms in presence of CFSs, increasing concentrations of 5, 12.5, 25 and 50 mg mL^{-1} of each reconstituted lyophilized CFS were added into the wells and kept for 24 h at 37 °C. After that, the microplate was washed three times with PBS, fixed with 99 % methanol for 15 min, and air-dried before adding 200 μL of 1 % *w/v* crystal violet (CV) (Sigma-Aldrich, St. Louis, MO, USA) to each well. Optical density of stained adherent cells was determined at 570 nm with an automatic microplate reader (SYNERGY H4 BioTek, BioTek Instruments, Agilent Technologies, Winooski, VT 05404, USA).

The eradication efficiency of the different CFSs against mature biofilm of S1 was tested by allowing biofilms formation for 24 h at 37°. After that, increasing concentrations of 15, 50, 125, 250 mg mL^{-1} of each tested lyophilized CFS were added and incubated for additional 24 h at 37 °C. Then, the planktonic cells were removed by PBS washing, and the residual biofilm cells were quantified by CV staining, as reported above. Biofilm reduction was calculated as [(OD_{570} of the control − OD_{570} of the treated)/OD_{570} of the control] × 100.

2.1.5. Antioxidant, cytotoxic and antiproliferative assays

The analysis of antioxidant activity of lyophilized CFSs was performed using diphenylpicrylhydrazyl (DPPH) free radical method, as reported by (Andrianto and Suzuki, 2015) - with slight modifications. Briefly, 800 μL of each lyophilized CFS solution (40 mg mL^{-1}) and 1 mL of 0.4 mM of DPPH solution were mixed with 20 % methanol aqueous solution and Tris-HCl buffer solution pH 7.4. The samples were stirred for 30 min in the dark at room temperature. The absorbance of the collected supernatants was measured at 517 nm. Ascorbic acid (40 μg mL^{-1}) was used as a comparison standard. The free radical scavenging activity (SA) was calculated as percentage by using the following formula:

$$SA = \left[1 - \frac{OD_{517}(\text{sample})}{OD_{517}(\text{blank})}\right] \times 100$$

To test for cytotoxic and antiproliferative effects of CFSs, the human keratinocyte cell line HaCaT and Human Caucasian colon adenocarcinoma Caco-2 cells obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) were employed. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich), supplemented with 10 % Fetal Bovine Serum, 1 % L-glutamine, and 1 % penicillin/streptomycin (Sigma Aldrich), in a humidified incubator at 37 °C and 5 % CO_2.

The effect of CFSs on HaCaT cells viability was assessed measuring the reduction of 3-(4,5-methylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) to formazan salts by the mitochondrial enzyme succinate dehydrogenase.

HaCaT cells were seeded in 100 μL of culture medium in 96-well plates at 1×10^4 cells per well and incubated for 24 h at 37 °C. After incubation, suitable volumes of CFSs stock solutions were added to obtain final concentrations of 5 mg mL^{-1}, 50 mg mL^{-1} and 250 mg mL^{-1} of lyophilized CFS and the cell culture incubated for additional 24 h at 37 °C. Then, MTT solution was added to each well and incubated at 37 °C for 4 h. The resulting formazan crystals were dissolved in DMSO and the absorbance was read at 570 nm in an ELISA reader.

For antiproliferative assay, after Caco-2 cells starvation, suitable volumes of CFSs stock solutions were added to obtain a concentration of 250 mg mL^{-1} of tested lyophilized CFS. After 24 h cells metabolic activity was measured by the MTT assay as described above.

2.1.6. Data analysis

GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com) was used for data analysis.

2.2. Metabolomic analysis

2.2.1. Sample preparation, derivatization and GC–MS analysis

Each CFS (100 μL) from single cultures of *L. lactis* subsp. *lactis* LAB2, *L. lactis* subsp. *hordniae* LAB1, *P. kudriavzevii* Y1 and from co-cultures of the three microorganisms was dried with a stream of nitrogen. Samples were resuspended in freshly prepared solution (50 μL, 15 g L^{-1}) of methoxylamine hydrochloride (Thermo Fisher Scientific) in pyridine (Fluka, Buchs, Switzerland) and the mixture was vortexed for 1 h at room temperature. Subsequently, 100 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (Fluka) were added and incubated for 2 min in a microwave oven at 180 W, as previously described (Félix et al., 2018; Guida et al., 2015). During the derivatization process, 0.100 g L^{-1} of naphthalene was added to each sample as an internal standard.

Observations on CFS samples define four classes (conditions) corresponding to three individual cultures and co-culture of the microorganisms and identified by labels 1LAB2, 2LAB1, 3PICHIA and 4CoCulture, respectively. Each class includes three or four technical replicates (GC–MS analysis) for each of three biological replicates, and the four classes totally include 47 observations.

For control and comparison purposes, GC–MS data were also collected by repeatedly subjecting the cultural broth (i.e., MRS) to the same analytical processes applied to CFSs.

Trimethylsilyl derivatives of metabolites were analyzed using an Agilent 6850 GC instrument (Milan, Italy) coupled to an Agilent 5973 Inert MS. For the separation an Agilent J&W HP-5 ms Ultra Inert GC capillary column (stationary phase: (5 %-Phenyl)-methylpolysiloxane; Length: 30 m; ID: 0.25 mm; Film Thickness: 0.25 μm) was housed in the GC oven. The following oven GC temperature program was employed: 70 °C for 1 min; 10 °C·min^{-1} until the column temperature reached 170 °C; 30 °C·min^{-1} until the column temperature reached 280 °C; 280 °C for 8 min. The solvent delay was set to 4 min. Helium was employed as carrier gas at a flow rate of 1 mL·min^{-1} and the GC injector was set at 250 °C in splitless mode. Measurements were conducted under electron impact (EI) ionization (70 eV) in full scan mode (*m/z* 35–550) at a frequency of 3.9 Hz. The EI ion source and quadrupole mass filter temperatures were kept, respectively, at 200 and 250 °C.

2.2.2. Data processing and statistical analysis

GC–MS data were deconvoluted using the National Institute of Standards and Technology (NIST) program Automated Mass Spectral Deconvolution and Identification System (AMDIS, www.amdis.net) which, for each GC–MS data file (i.e., for each observation), generates a target (.FIN) and a component (.ELU) result file. The full set of 47 .FIN and 47 .ELU files (corresponding to 47 observations on cultures and co-culture of the three microorganisms) were processed by our in-house

program developed in MATLAB R2023a (Mathworks, Natick, MA, USA, www.mathworks.com). First, our program tracked conserved metabolites throughout the observations creating a matrix (which we will refer to as Integrated Signal matrix or IS matrix) with rows corresponding to conserved components and columns corresponding to the 47 observations. Technically, in this context, a conserved component is one which signal consistently persists in replicated samples (at least in 75 % of the observations in one class or condition). Each cell of the IS matrix contains a value which corresponds to the integrated signal, or abundance, attributed by AMDIS to the component identified by the row label in the observation identified by the column label. Components were identified by matching their deconvoluted EI mass spectrum at 70 eV with those collected in the NIST20 mass spectral library (https://www.nist.gov/srd/nist-standard-reference-database-1a) and possibly verified against the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/). The identification was further supported by Kovats retention index (RI) calculated for each component by the Kovats equation using chromatographic data from a standard *n*-alkane mixture in the range C7–C40 (Sigma-Aldrich, Saint Louis, MO, USA) analyzed under the same conditions. After identification, the IS matrix included 81 rows (each corresponding to an identified metabolite). Before multivariate and univariate analyses, in order to mitigate the effect of random experimental and instrumental factors which may affect measurements, the abundances in each column (observation) of the IS matrix were first normalized with respect to the corresponding signal of the internal standard added during the preparation of the samples (i.e., 0.100 g L^{-1} naphthalene).

3. Results

The following subsections present the results of antimicrobial, antibiofilm, antioxidant, cytotoxic and antiproliferative biological tests performed on CFSs from single cultures and co-cultures of the considered microorganisms, as well as the results of supervised and unsupervised analyses of the metabolomic dataset collected in the IS matrix.

3.1. In Vitro Biological Assays

From the single and co-cultures of *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1 and *L. lactis* subsp. *lactis* LAB2, cell-free supernatants (CFSs) were prepared and lyophilized. Lyophilized CFSs were redissolved in 2 % DMSO as reported in Material and Methods section and used to investigate their antimicrobial, antibiofilm, antioxidant, cytotoxic and antiproliferative activities.

3.1.1. Antimicrobial and antibiofilm activities

Table S2 in the Supporting Information shows the minimum inhibitory concentration (MIC) of the different CFSs against a panel of enteric pathogens, expressed as the lowest concentration in mg mL^{-1} of lyophilized CFS which prevents pathogen growth. As can be seen, all the CFSs under examination were active against *Salmonella* sp. S1 and showed the same MIC of 50 mg mL^{-1}. On the contrary, all CFSs were inactive against *L. monocytogenes* for which it has not been possible to determine the MIC. The antimicrobial effects of CFSs against the other enteric strains were lower. MIC for the two *E. coli* strains reached 250 mg mL^{-1} for CFSs from LAB1 and LAB2 cultures and was higher than 250 mg mL^{-1} for CFSs from yeast culture and co-culture.

Building on the promising antimicrobial activity of CFSs against *Salmonella* sp. S1, we investigated the ability of CFSs to prevent biofilm formation and eradicate mature biofilms of this pathogen. *Salmonella* sp. can form biofilms on various inert or living surfaces, an ability that contributes to its resistance and persistence in both host and non-host environments (Steenackers et al., 2012) and we have confirmed that this is also the case for *Salmonella* sp. S1.

Biofilm formation was challenged by stepwise increasing concentration of lyophilized CFSs from 5 to 50 mg mL^{-1}. As showed in Fig. 1, biofilm inhibition follows a dose-dependent pattern and complete biofilm inhibition is reached at MIC concentrations for all CFSs. Sub-MIC concentrations of lyophilized CFSs in the range 12.5–25 mg mL^{-1} inhibited biofilm formation up to about 30–50 % but, most significantly, differences between different CFSs are very limited at any given concentration level.

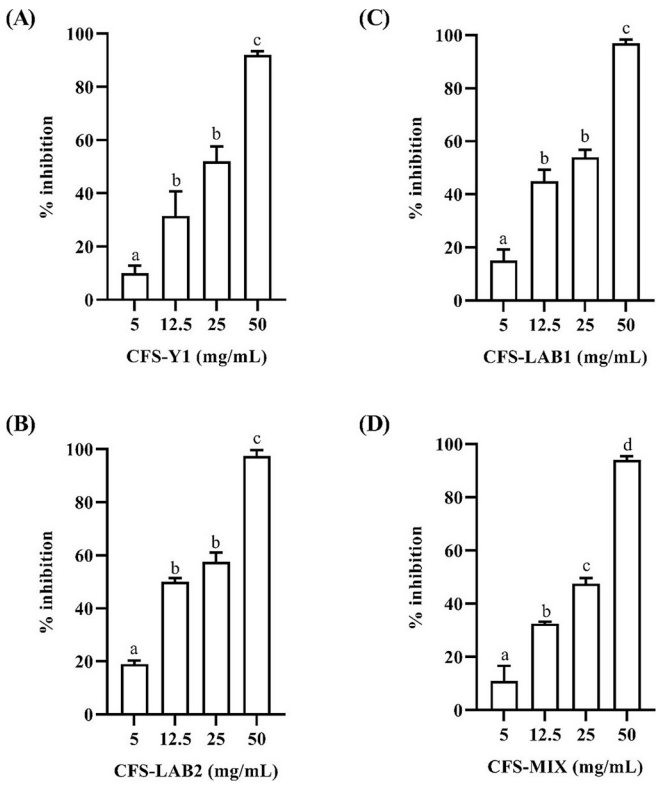

Fig. 1. Inhibition of biofilm of *Salmonella* sp. S1 by lyophilized cell free supernatants (CFSs) of single cultures of: (A) *P. kudriavzevii* Y1; (B) *L. lactis* subsp. *lactis* LAB2; (C) *L. lactis* subsp. *hordniae* LAB1; (D) co-culture of the three microorganisms. Biofilms were quantified after 24 h growth at 37 °C with the colorimetric CV method. Data represent the mean ± SD of three independent experiments; values with dissimilar letters are significantly different from each other ($p < 0.05$, Tukey's multi comparison test).

To test for biofilm eradication ability of CFSs, *Salmonella* sp. S1 biofilms, grown for 24 h at 37 °C in 96-wells microtiter plate, were exposed to concentrations of lyophilized CFSs ranging from 15 to 250 mg L^{-1} for another 24 h at 37 °C. As can be seen from supplementary Fig. S1, CFSs are not only capable of inhibiting biofilm formation, but are also effective in removing established biofilms of *Salmonella* sp. S1 in a dose-dependent manner. Most significantly, we see that CFS from *P. kudriavzevii* culture, at any given concentration level, has a lower capacity to eradicate *Salmonella* sp. biofilms compared to CFSs from cultures of lactic acid bacteria which expose the highest eradication rate; CFSs from co-culture degrade *Salmonella* sp. S1 biofilms at a rate higher than CFSs from yeast cultures but lower than CFS from LAB1 and LAB2 bacteria which have very similar effects.

3.1.2. Antioxidant, cytotoxic and antiproliferative activities

To evaluate the antioxidant activity of lyophilized CFSs from single and co-cultures of *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1, *L. lactis* subsp. *lactis* LAB2, solutions of the corresponding lyophilized CFSs at 40 mg mL^{-1} concentration were prepared and tested using diphenylpicrylhydrazyl (DPPH) free radical method (see Materials and Methods section). Ascorbic acid (40 μg mL^{-1}), which is well-known for its antioxidant activity, was used as a comparison standard. The results exposed in Fig. 2 show that CFSs from co-culture exhibited a superior

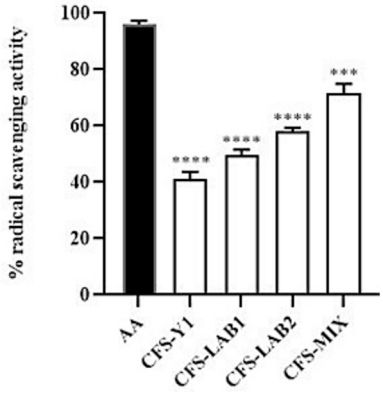

Fig. 2. Free radical scavenging activity (SA) of lyophilized cell free supernatants (CFSs) from single and co-cultures of *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1, *L. lactis* subsp. *lactis* LAB2. Lyophilized CFS solutions (40 mg mL^{-1}) were tested by using the diphenylpicrylhydrazyl (DPPH) free radical method. 40 μg mL^{-1} ascorbic acid (AA) is used as a comparison standard. Scavenging activity was calculated from the formula SA = 100 × [1 – OD_{517}(sample)/OD_{517}(blank)]. Results are the mean ± SD of three independent experiments. *** = $p < 0.001$, **** = $p < 0.0001$ (Dunnett's multi comparison test).

free radical scavenging activity (~75 %) compared to CFSs from single cultures of microorganisms.

The cytotoxic activity of lyophilized CFSs of single and co-cultures of *P. kudriavzevii* Y1, *L. lactis* subsp. *hordniae* LAB1, *L. lactis* subsp. *lactis* LAB2 was evaluated on human keratinocyte cell line (HaCaT), a spontaneously immortalized, non-tumorigenic cell line derived from adult human skin, using the colorimetric MTT assay. Cells were exposed to concentrations of lyophilized CFSs ranging from 5 to 250 mg mL^{-1} for 24 h at 37 °C. Our results, expressed as the average percentage of cell viability relative to the viability of untreated cells (control) are shown in the supplementary Fig. S2A and clearly demonstrate that, at the concentrations tested, CFSs caused no significant cell damage.

From supplementary Fig. S2B, we see that lyophilized CFSs can decrease survival among colon cancer cells. Indeed, a reduction of cell viability of about 30 %, 35 %, 40 % and 30 % is observed for CFSs from individual cultures of Y1, LAB1, LAB2 and co-culture, respectively, after 24 h of exposure to a concentration of 250 mg mL^{-1} of each lyophilized CFS.

3.2. Metabolomic analysis

To investigate the metabolic profiles of single cultures and co-cultures of *L. lactis* subsp. *lactis* LAB2, *L. lactis* subsp. *hordniae* LAB1 and *P. kudriavzevii* Y1, cell free supernatants (CFSs) were analyzed via GC–MS, after methoxymation and trimethylsilylation. A matrix (IS matrix) was created from the raw GC–MS data, the columns of which correspond to observations grouped into four classes labelled as 1LAB2, 2LAB1, 3PICHIA, 4CoCulture, and which is the basis of all subsequent evaluations (see Materials and Methods section).

Identified metabolites, corresponding to rows of the IS matrix, are listed in Table 1 from which we see that detected metabolites belong to diverse groups of natural products, e.g., carbohydrates, polyols, amino acids, polyamines and lipids. In addition, several products of the secondary metabolism of microorganisms were detected (e.g., tyrosol, phenylethyl alcohol, 3-phenyllactic acid, tryptophol, putrescine, cadaverine).

The scatter plot in supplementary Fig. S3 graphically extends the data in Table 1 by reporting, the log10 of average abundances of each metabolite in each of the four classes (i.e., 1LAB2, 2LAB1, 3PICHIA, 4CoCulture). The primary purpose of Fig. S3 is to show by simple visual inspection that measured abundances of detected metabolites span

Table 1
Identified extracellular metabolites in cell-free supernatants from individual cultures of *Lactococcus lactis* subsp. *lactis* LAB2, *Lactococcus lactis* subsp. *hordniae* LAB1, *P. kudriavzevii* Y1, and co-culture of the three microorganisms. RT represents retention time (minutes), RI represents Kovats retention index and TMS is the trimethylsilyl function, $(CH_3)_3Si$-. The number in the first column is used in subsequent figures as a substitute for the full name of each metabolite. Metabolites are listed in an order set by the algorithm employed for the subsequent unsupervised analysis of the dataset.

#	Name	RI	RT
1	2,3-Butanediol, 2TMS	1065	4.7915
2	Tryptophan, 3TMS	2252	14.2914
3	Pro-Ala, 2TMS	1813	12.5809
4	Asparagine, 3TMS	1687	11.8911
5	Aspartic acid, 3TMS	1539	10.7787
6	Serine, 3TMS	1374	8.8901
7	Threonine, 3TMS	1401	9.241
8	Gluconic acid, 6TMS	2050	13.5626
9	Trehalose 8TMS	2814	17.4362
10	Lysine, 3TMS	1720	12.0868
11	Pyruvic acid, enol, 2TMS	1110	5.3915
12	Xylitol, 5TMS	1747	12.2315
13	Allofuranose, 5TMS	1876	12.8696
14	Glycerol, 3TMS	1288	7.7612
15	Allopyranose, 5TMS	2022	13.4596
16	Glucose, methyloxime, 5TMS	1932	13.1091
17	5-Hydroxy-2-(hydroxymethyl)pyridine, 2TMS	1550	10.8752
18	Arabinono-1,4-lactone, 3TMS	1656	11.687
19	3-Hydroxy-2,3-dihydromaltol, 2TMS	1466	10.0218
20	Deoxyfructosazine, 7TMS	2695	16.4888
21	Oleic Acid, TMS	2220	14.1709
22	1-Glycerophosphate, 4TMS	1791	12.4732
23	Citric acid, 4TMS	1852	12.7603
24	Glucopyranose, 1-O-(3-O-(2-methylbutanoyl)-glucopyranosyl), 7TMS	2657	16.243
25	Erythronic acid, 4TMS	1566	11.016
26	Lactic Acid, 2TMS	1088	5.0955
27	Fumaric acid, 2TMS	1352	8.6052
28	Glyceric acid, 3TMS	1343	8.4883
29	2-Aminomalonic acid, 3TMS	1484	10.2465
30	Malic acid, 3TMS	1503	10.4687
31	Glycolic acid, 2TMS	1098	5.2331
32	Isoleucine, 2TMS	1307	8.0072
33	Phenylalanine, 2TMS	1645	11.615
34	Leucine, 2TMS	1283	7.6982
35	Valine, 2TMS	1229	6.9677
36	Guanine, 3 TMS	2148	13.915
37	Methionine, 2TMS	1533	10.7322
38	cycloOrnithine, 2TMS	1469	10.0688
39	Alanine, 2TMS	1124	5.575
40	Proline, 2TMS	1312	8.0825
41	2-Hydroxyisocaproic acid, 2TMS	1250	7.2585
42	Glycine, 2TMS	1136	5.7389
43	Glutamic acid, 3TMS	1636	11.5569
44	Ornithine, 3TMS	1628	11.5032
45	Succinic acid, 2TMS	1327	8.2681
46	Tyrosine, 3TMS	1975	13.2804
47	Cyclo-(Ala-Leu), 2TMS	1906	13.0049
48	Glucose, 5TMS	1998	13.3702
49	Cadaverine, 4TMS	1857	12.7803
50	Putrescine, 4TMS	1757	12.2891
51	Hydroxyglutaric acid, 3TMS	1586	11.1934
52	3-Deoxyhexonic acid, 5TMS	1885	12.9109
53	3-Phenyllactic acid, 2TMS	1597	11.2928
54	Tyrosol, 2TMS	1581	11.1493
55	2-Hydroxy-2-methylbutyric acid, 2TMS	1179	6.3102
56	3,4-Dihydroxybutanoic acid, 3TMS	1424	9.5152
57	Thymine, 2TMS	1376	8.9106
58	5-Hydroxylysine, 4TMS	1871	12.8457
59	Erythritol, 4TMS	1529	10.6946
60	Phenylethyl Alcohol, TMS	1237	7.0795
61	Uracil, 2TMS	1350	8.5718
62	Mannobiose, 8TMS	2678	16.3796
63	Tryptophol, 2TMS	1917	13.0497
64	Pantothenic acid 3TMS	2014	13.4327
65	Urea, 2TMS	1279	7.6378
66	1,2,3-Butanetriol, 3TMS	1305	7.9877

(*continued on next page*)

Table 1 (*continued*)

#	Name	RI	RT
67	Xanthine, 3TMS	2045	13.5444
68	5-Methylhydantoin, 2TMS	1413	9.3893
69	Hydroxyurea, 2TMS	1157	6.0097
70	Myo-Inositol, 6TMS	2132	13.8584
71	Ribonic acid, 5 TMS	1808	12.5568
72	2-Deoxy-erythro-pentonic acid, 4TMS	1677	11.8276
73	Cyclo-(Gly-Gly), 2TMS	1435	9.6482
74	Phosphate, 3TMS	1290	7.7831
75	5-Oxoproline, 2TMS	1544	10.821
76	3-Aminopiperidine-2,6-dione, 2TMS	1621	11.4532
77	Dimethylglycine, TMS	1028	4.3033
78	2,3-Dihydroxy-2-methylpropanoic acid, 3TMS	1333	8.3573
79	Cyclo-(Gly-Pro), TMS	1750	12.251
80	Cyclo-(Ala-Gly), 2TMS	1393	9.14
81	2-Amino-1-methylimidazol-4-ol, 3TMS	1575	11.0927

several orders of magnitude. For example, from Fig. S3 we briefly see that the level of lactic acid (#26) in the CFSs from cultures of LAB bacteria is about three orders of magnitude higher than that in the CFS from *P. kudriavzevii* culture, while the level of lactic acid in the co-culture of the three microorganisms is intermediate between its level in cultures of LAB bacteria and that in the yeast culture. Furthermore, Fig. S3 also allows comparison of abundances of different metabolites. For example, we easily see that abundance of lactic acid in CFSs from LAB2 and LAB1 cultures is, with few exceptions (e.g., phosphate, #74), higher than that of any other identified metabolite.

3.2.1. Unsupervised data analysis

The IS matrix incorporates observations which nominally belong to four different classes (i.e., 1LAB2, 2LAB1, 3PICHIA and 4CoCulture). It is then expected that, within biological and technical variability, observations in the same class are similar to each other. To verify this expectation and to identify observations which deviate from the expected pattern, data in the IS matrix were subjected to Hierarchical Clustering Analysis (HCA). However, before HCA, in order to make metabolites (variables) equally important, regardless of their concentrations in samples, for each metabolite, *i*, a standard deviation (s_i) was calculated over the full array of abundances representing the metabolite in a row of the IS matrix and data in each row were scaled by the corresponding standard deviation. HCA results are presented as a cluster tree (dendrogram) and associated heatmap in Fig. 3.

In a hierarchical cluster tree, any two objects are eventually linked together at some level. Objects are either observations in the original data set or groups of observations created by the HCA algorithm. The links between objects are represented as upside-down U-shaped lines. The height of the link represents the distance (dissimilarity) between the two objects. This height is known as the *cophenetic distance* between the two objects and can be read on the ordinate of the dendrogram. A link whose height differs significantly from the height of the underlying links indicates that the objects joined at this level in the cluster tree are much further apart and is called an inconsistent link. The relative inconsistency of each link in a hierarchical cluster tree can be quantified and expressed as a link inconsistency value. Links joining distinct clusters have relatively high link inconsistency values; links joining indistinct clusters have relatively low link inconsistency values. Furthermore, a hierarchical tree can be associated with a tree cophenetic coefficient that measures how closely the tree represents natural dissimilarities between observations. The magnitude of this value should be very close to 1 for a high-quality clustering solution. Since the number of clusters is not a result of HCA analysis, observations are allocated to clusters and a clustering solution is created by drawing a horizontal line through the dendrogram at a selected height. Observations that are joined together below the line are in clusters.

From the dendrogram in Fig. 3 we see that a horizontal line drawn at a cophenetic distance of about 20 creates four clusters each containing observations in a single class. This proves that observations in a given class are much more similar to each other than to observations in other classes. From the dendrogram we also see that observations in class 3PICHIA are more dissimilar from observations in class 4CoCulture than are observations in class 1LAB2 from observations in class 2LAB1. Furthermore, the 24 observations in classes 3PICHIA and 4CoCulture appear very much dissimilar from the 23 observations in classes 1LAB2 and 2LAB1. This can also be deduced from the heatmap which is a visual representation of metabolites levels across clustered observations and helps link the clustering results exposed in the dendrogram to the variables or groups of variables (metabolites) in the IS matrix which can be useful to pick up variables that are most important for the separation between classes. In fact, the HCA algorithm automatically sets the metabolites in an order that facilitates the interpretation of the associated heatmap. For example, in line with the above claims, we easily see that the heatmap slices corresponding to clusters 1 and 2 have a strikingly similar colour to each other, while the slices corresponding to clusters 3 and 4 appear to have a more different colour.

In the HCA analysis above, a somewhat arbitrary step concerns the height at which the hierarchical tree was trimmed to create a clustering solution with four clusters. The Silhouette Plot in supplementary Fig. S4 validates this choice by showing that it produces a valid clustering solution since all observations have high silhouette values (Kaufman & Rousseeuw, 2009). Further support to the clustering solution from HCA analysis can also be obtained by subjecting data in the IS matrix to Principal Component Analysis (PCA). The PCA score plot in supplementary Fig. S5 is clearly in full agreement with the clustering solution obtained from HCA analysis by cutting the cluster tree at a height that creates four clusters.

3.2.2. Supervised data analysis

The MRS broth is a rich culture medium which contains an assortment of biogenic substances (e.g., amino acids and proteins). Therefore, to identify metabolites actually excreted by the cultured microorganisms, we subjected the MRS broth to the same analytical procedure employed for the CFSs from cultures of the microorganisms. Thus, the MRS broth observations define a further class, which will be referred to as the 5MRS class, in addition to the four culture classes which incorporate observations on CFSs from cultures of the microorganisms and, in the supervised analysis, we will consider five labelled classes (i.e., 1LAB2, 2LAB1, 3PICHIA, 4CoCulture and 5MRS). Supervised analysis is performed by univariate pairwise comparisons of metabolite levels in pairs of classes that allow to gain insights into the biological significance of metabolites and their interactions by mapping and comparing the quantitative trajectories of metabolites across the space of classes.

Therefore, from the set of five classes mentioned above we extracted 7 pairs (i.e., 1LAB2/5MRS; 2LAB1/5MRS; 3PICHIA/5MRS; 4CoCulture/5MRS; 4CoCulture/1LAB2; 4CoCulture/2LAB1; 4CoCulture/3PICHIA) and compared, one by one, the metabolite levels in each of the 7 selected pairs. In any pairwise class comparison, we will refer to the class before the slash symbol as the “numerator class” and to the class after the slash as the “denominator class”.

In any pairwise class comparison, for each metabolite we calculated a Fold Change (FC) by taking the ratio of its average abundances in the numerator class to its average abundance in the denominator class. Furthermore, we subjected the array of abundance values representing a metabolite in each of the two classes to Student’s *t*-test for equal means. After that, the average abundances of a given metabolite were considered significantly different in the two compared classes if the associated *p*-Value was lower than 0.05 (i.e., the significance threshold is set to 0.05) and the FC was higher than 2 (upregulated in the numerator class) or lower than 0.5 (downregulated in the numerator class). Results of the 7 pairwise class comparisons are presented in Figs. 4 and 5 in the form of error bar line plots. Specifically, plots in Figs. 4A-D present results from the comparison of each of the four culture classes with the MRS broth; and plots in Figs. 5A-C collect results of the comparison of CFSs from the

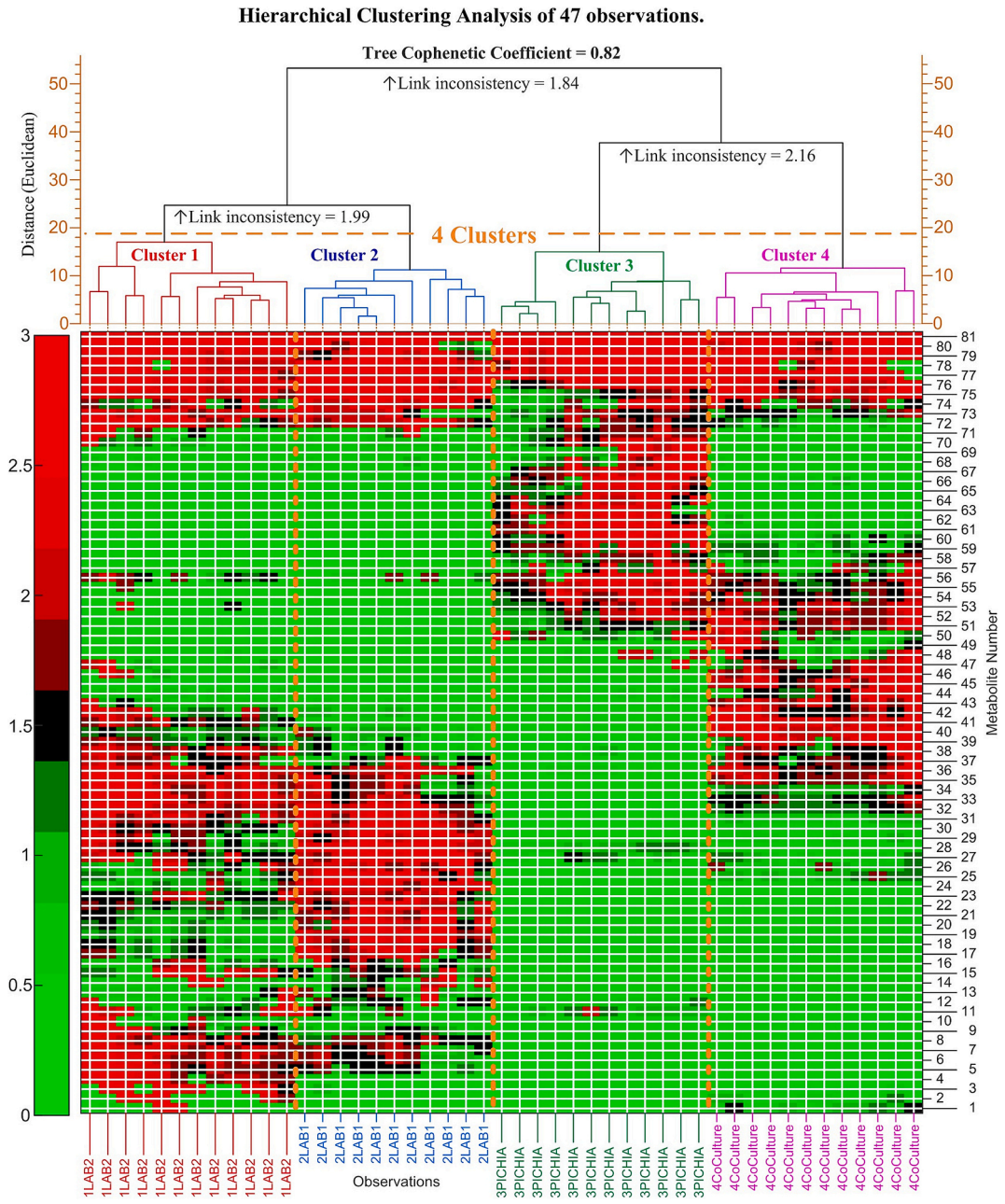

Fig. 3. Clustergram describing Hierarchical Clustering Analysis (HCA) of 47 observations on CFSs from *L. lactis* subsp. *lactis* LAB2, *L. lactis* subsp. *hordniae* LAB1, *P. kudriavzevii* Y1 and from co-culture of the three microorganisms. HCA analysis was performed setting the distance metric to Euclidean and the linkage parameter to Ward. Metabolites are identified by the numerical code in Table 1.

co-culture with CFSs from each of the individual cultures of the microorganisms.

Each plot contains two polyline connecting markers (points) representing average abundances of the metabolites in the two compared classes. To extend the significance of the line graphs, each metabolite has been associated with an explanatory label, shown at the top of each graph, that encode information about the FC and displays the *p*-Value, which is reported after the number sign (i.e., #) of each label. Formally, FC = Inf for a metabolite not detected in the denominator class or FC = 0 for a metabolite not detected in numerator class and such metabolites are distinguished by orange labels containing the symbols UPz and DWz, respectively.

Note that, since metabolite abundances can differ by several orders of magnitude, to improve the comprehensibility of plots in Figs. 4 and 5, the average abundances of each metabolite have been scaled by the standard deviation of its abundances in the two compared classes. Obviously, for any given metabolite, scaling does not change the ratio of mean abundances and, consequently, we can graphically evaluate the FC by reading the mean abundances of the metabolite (Y-coordinate) in each of the two compared classes.

The number of metabolites which levels are not significantly different in the two compared classes is used to evaluate the similarity of the two classes by computing a similarity index which is reported in the title of each plot. The similarity index is defined as the ratio of the number of non-significantly different metabolites and the total number of metabolites detected in the two compared classes: the similarity of the two compared classes increases as the similarity index increases.

Therefore, from the plots in Fig. 4 it is possible to extract briefly various general information on the biosynthetic activity of the microorganisms in individual cultures and co-culture. Considering Fig. 4A and

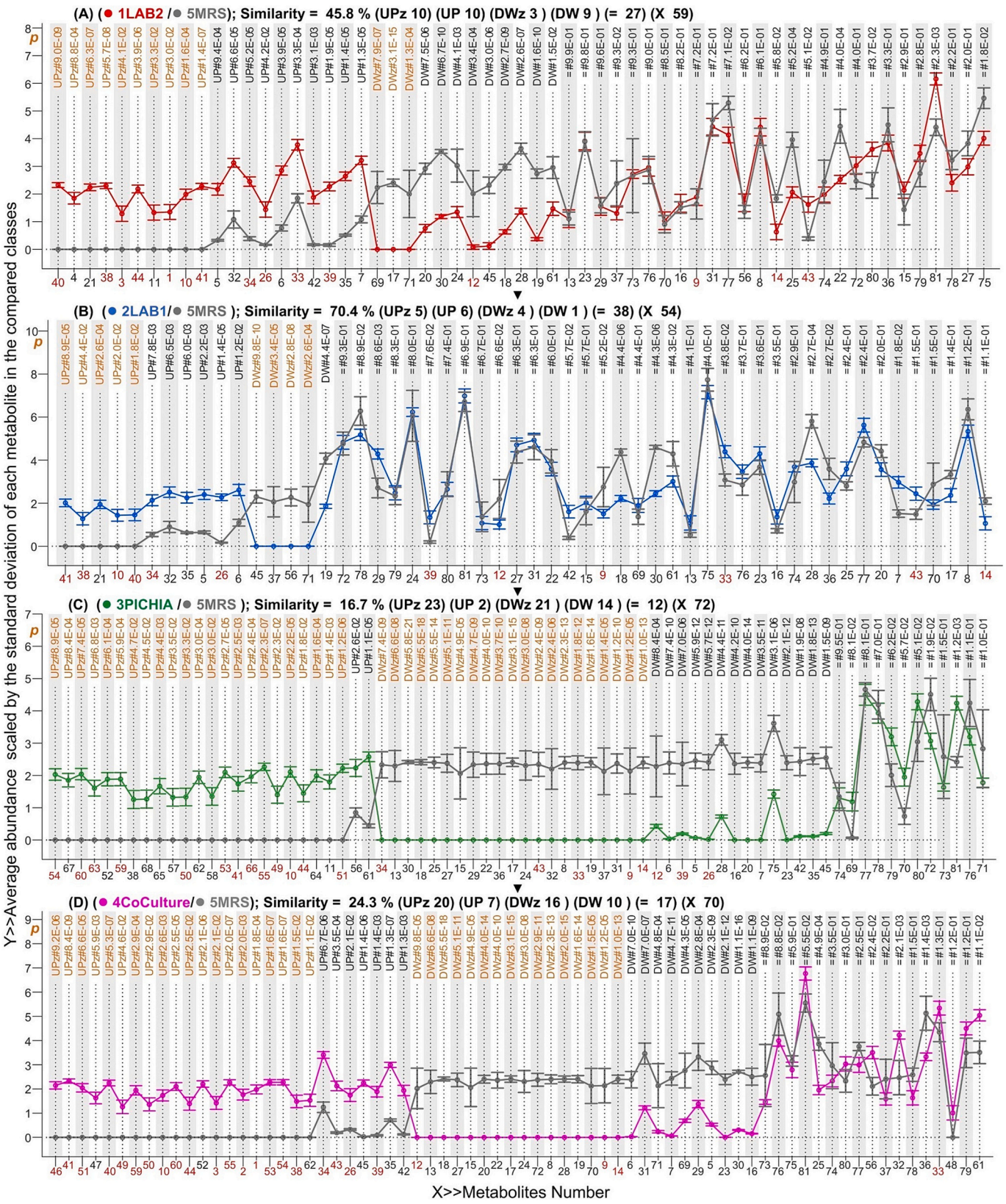

Fig. 4. Pairwise comparisons of metabolites levels between classes: (A) 1LAB2/5MRS; (B) 2LAB1/5MRS; (C) 3PICHIA/5MRS; (D) 4CoCulture/5MRS. Labels UPz and UP (upregulated in CFSs from cultures with respect to MRS broth) and DWz and DW (downregulated in CFSs from cultures with respect to MRS broth) indicate metabolites differentially expressed in the two compared classes. An orange font has been used for the labels corresponding to UPz and DWz metabolites. Error bars represent the standard deviation of the mean of replicated measurements. Data have been scaled by the standard deviation of each metabolite in the two compared classes. Metabolites are identified by the numerical code in Table 1 and, to facilitate comparisons, are listed in an order that minimizes intersections between the two polylines.

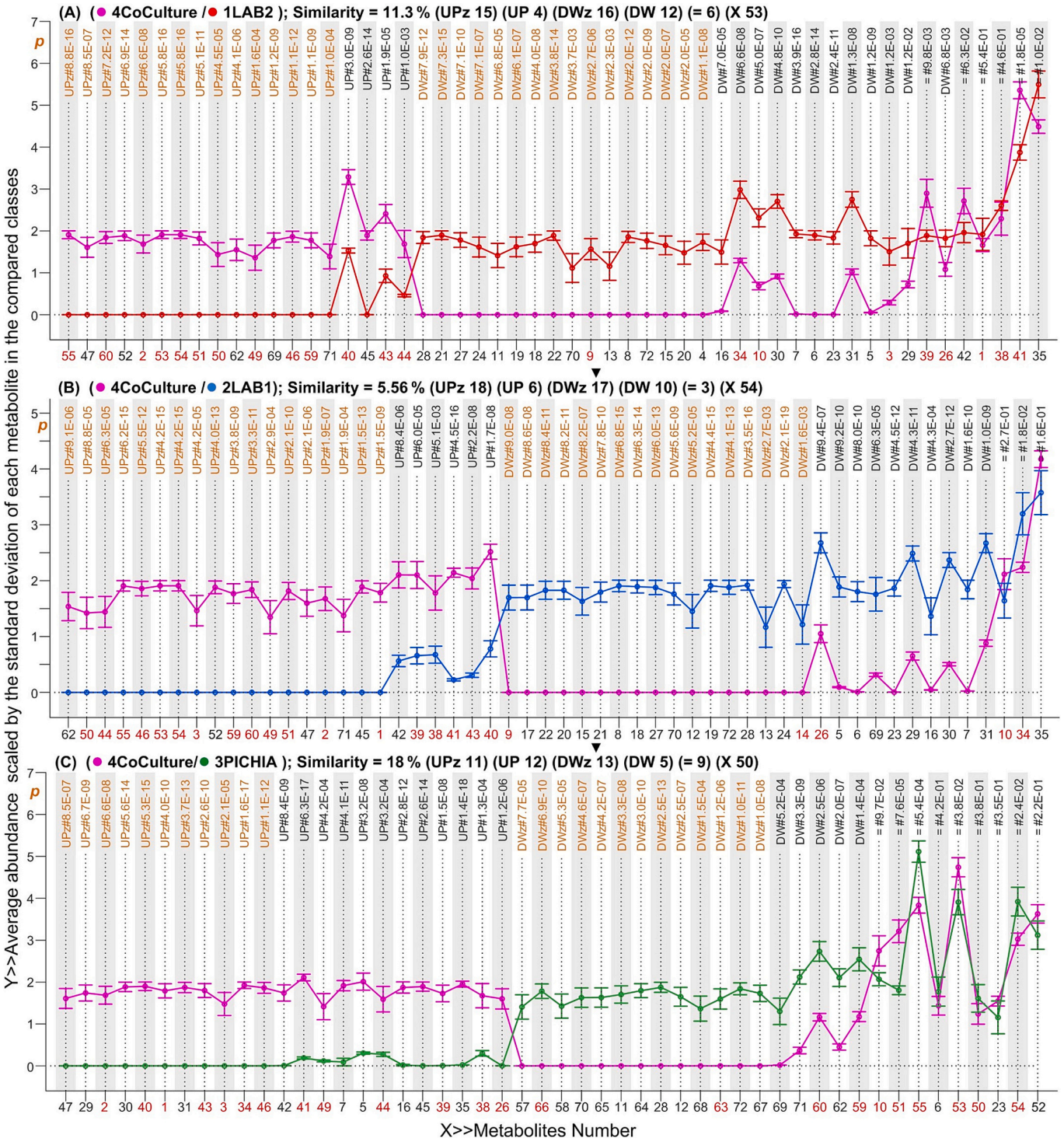

Fig. 5. Pairwise comparisons of metabolites levels between classes: (A) 4CoCulture/1LAB2; (B) 4CoCulture/2LAB1; (C) 4CoCulture/3PICHIA. Labels UPz and UP (upregulated in co-culture with respect to individual culture) and DWz and DW (downregulated in co-culture with respect to individual culture) indicate metabolites differentially expressed in the two compared classes. An orange font has been used for the labels corresponding to the UPz and DWz metabolites. Error bars represent the standard deviation of the mean of replicated measurements. Data have been scaled by the standard deviation of each metabolite in the two compared classes. Metabolites are identified by the numerical code in Table 1 and, to facilitate comparisons, are listed in an order that minimizes intersections between the two polylines.

B we immediately see that LAB2 is metabolically more active than LAB1. Indeed, the number of UPz and UP metabolites is greater in Fig. 4A than in Fig. 4B. Consequently, we read a similarity index of 45.8 % in Fig. 4A and 70.4 % in Fig. 4B which confirm that LAB2 has a superior ability to modify MRS broth composition than LAB1. However, the metabolic activity of both LAB2 and LAB1 bacteria is much lower than the metabolic activity of *P. kudriavzevii* in the individual yeast culture since in Fig. 4C we read a similarity index of only 16.7 %. The similarity index of the CFS from co-culture and the MRS broth in Fig. 4D is 24.3 % from which we can deduce that intense metabolic activity is present in the co-culture as can also be deduced from visual inspection of the diverging trajectory of the two polylines in Fig. 4D.

To facilitate discussion, it is useful to summarize the information in Fig. 4, by associating to each metabolite an array of "coordinates" which specify its presence/absence in each of the five classes. For instance, a metabolite which has been detected in all five classes will have coordinates {1, 2, 3, 4, 5} where numbers from 1 to 5 specify ordinately the five classes 1LAB2, 2LAB1, 3PICHIA, 4CoCulture and 5MRS. A metabolite which has not been detected in class 1LAB2 will have coordinates {0, 2, 3, 4, 5}. Vice versa, a metabolite with coordinates {1, 2, 0, 4, 0} is a metabolite which has been detected in classes 1LAB2, 2LAB1 and 4CoCulture but has not been detected in classes 3PICHIA and 5MRS. The coordinates of a specified metabolite can be determined from Fig. 4. For example, by parsing Fig. 4 for putrescine (#50) we see that its coordinates are {0, 0, 3, 4, 0} which means that putrescine has not been detected in classes 1LAB2, 2LAB1 and 5MRS but has been detected in classes 3PICHIA and 4CoCulture. In fact, putrescine only appears in plots of Fig. 4C and D where it is listed as an UPz metabolite. Similarly, the dipeptide Pro-Ala (#3) has coordinates {1,0,0,4,0} implying that it has only been detected in classes 1LAB2 and 4CoCulture since it is listed as an UPz metabolite in plots of Fig. 4A and D and it is not listed either in Fig. 4B or in Fig. 4C.

The coordinates of all metabolites in Table 1, determined by parsing Fig. 4 metabolite by metabolite, are listed in Table S3 since they will be mentioned in the subsequent discussion.

The results reported in Fig. 4D allow to reduce the number of metabolites to be considered for further analysis from 81 to 64 by excluding the last 17 metabolites on the right of Fig. 4D which are present in equal amounts in the co-culture and in the MRS broth. This strategy simplifies data analysis without losing information since it is obviously irrelevant for our purposes to consider substances that are not influenced in any way by the biosynthetic activity of the microorganisms in the co-culture (although they may be present in the chemical environment in which the three microorganisms grew). On this basis, only 64 metabolites are considered for the construction of plots in Fig. 5.

Several interesting inferences can be made concerning the metabolic activity of the three organisms in the co-culture by combining information in Figs. 4 and 5.

For example, as we have shown above, from Fig. 4 it results that metabolite #50 (i.e., putrescine) has coordinates {0, 0, 3, 4, 0). This means that putrescine has only been detected in CFSs from the yeast culture and from co-culture. Thus, since there is no evidence from our data that lactic acid bacteria can produce putrescine, we may infer that in the co-culture putrescine is produced by *P. kudriavzevii*. From Fig. 5C, we see that the label associated with putrescine contains the equal sign and then, its level is not significantly different in the yeast culture and co-culture. Thus, we may conclude that in the co-culture putrescine is produced by *P. kudriavzevii* and its production is unaffected when the yeast is co-cultured with LAB2 and LAB1 bacteria.

In contrast, consider now that the dipeptide Pro-Ala (#3) is likely produced in the co-culture by LAB1 bacterium since it has coordinates {1,0,0,4,0}. Obviously, the dipeptide Pro-Ala appears in the graph in Fig. 5A where it is listed as a DW metabolite (implying that it was detected in the co-culture at a lower level than in the individual LAB2 culture). Combining these facts, we can infer that Ala-Pro is produced by the LAB2 bacterium, but its production decreases when the LAB2 bacterium is co-cultured with the yeast and the LAB1 bacterium.

From the examples above, it can be perceived how by parsing Figs. 4 and 5 metabolite by metabolite, it is possible to highlight a list of metabolites whose biological properties and trajectory in the space of classes are particularly relevant to our purposes. The metabolites in this list are marked in Figs. 4 and 5 using a red font for their code number, because they will be mentioned in the subsequent discussion, while Fig. S6 provides the structures and a brief summary of the biological activities of the most significant metabolites.

4. Discussion

Probiotic microorganisms produce a variety of metabolites which are considered to be central for their capacity to promote health benefits. Whereas it is traditionally assumed that probiotic microorganisms must be alive during administration, new evidence shows that administration of probiotic-derived metabolites (postbiotics) may be sufficient to promote positive effects on the human health (Ruiz et al., 2014).

In this study the postbiotic capabilities of three probiotics candidates isolated from traditional homemade kefir have been investigated. In this respect, cell free supernatants (CFSs) from single and co-cultures of *L. lactis* subsp. *lactis* LAB2, *L. lactis* subsp. *hordniae* LAB1 and *P. kudriavzevii* Y1 were shown to possess interesting antimicrobial, antibiofilm, antioxidant, cytotoxic and antiproliferative activities. The subsequent application of a GC–MS metabolomic footprint approach, to explore the biosynthetic ability of these microorganisms allowed us to investigate the potential relationships between the metabolite production and the observed CFS bioactivities. Moreover, metabolomic data shed some light on the role of metabolites into the interspecies interactions in co-cultures of the probiotics.

As it is well known, lactic acid bacteria cannot completely obtain the energy embodied in a glucose molecule because they do not have a functional respiratory chain (Pessione, 2012; Vinderola et al., 2019) and, therefore, are unable to dispose of electrons (e^-) released by the complete oxidation of glucose to carbon dioxide ($C_6H_{12}O_6$(glucose) + $6H_2O \leftrightharpoons 6CO_2$(carbon dioxide) + $24H^+ + 24e^-$). However, LAB overcome this problem by decomposing, via glycolysis, a glucose molecule into two lactic acid molecules which are then excreted. Although the chemical bonds of glucose are rearranged into two molecules of lactic acid ($C_6H_{12}O_6$(glucose) $\leftrightharpoons 2C_3H_6O_3$(lactic acid)), this reaction is not an oxidation of glucose and lactic acid is simply half glucose (Salvatore & Salvatore, 2023). Nevertheless, this is an energy-expensive strategy since simply splitting glucose into two halves only makes available to the cell a very small fraction of the energy that could be obtained from a glucose molecule. Therefore, lactic acid is an energy-rich molecule and any microorganism capable of metabolizing and extracting energy from lactic acid freed by the lactic acid bacteria metabolism of glucose would benefit from their presence in the ecosystem. On the other side, since excessive lactic acid tends to decrease the pH of the culture broth and can be harmful to lactic acid bacteria themselves, LAB will benefit by the presence of microorganisms which mitigate the buildup of lactic acid in the chemical environment. Our data showed a dramatic increase in the level of lactic acid (#26) in individual cultures of LAB2 and LAB1 compared to MRS broth, which also contains lactic acid which has coordinates {1,2,3,4,5} (see supplementary Table S3 and Fig. S3). On the other hand, from Fig. 4C it seems that *P. kudriavzevii* can metabolize lactic acid present in the MRS medium, since lactic acid is downregulated in class 3PICHIA with respect to class 5MRS (FC = 0.009). Moreover, from Fig. 5 or from Fig. S3 we see that lactic acid level in co-culture is intermediate between its level in LAB cultures and its level in the yeast culture. Hence, an obvious explanation for this is that *P. kudriavzevii* consumes some of the lactic acid produced by LAB. However, the yeast absorbs only a fraction of the lactic acid produced by LAB2 and LAB1, so we observe an upregulation of lactic acid in the co-culture compared to the single *P. kudriavzevii* culture. We note that the idea that yeasts can use lactate as an energy and/or carbon source has already been put forward to explain the observation that *Lactobacillus* spp. fail to decrease the pH of the culture broth when co-cultured with *C. albicans* (Zangl et al., 2020). The processes through which lactate metabolism occurs can be different depending on the yeast and growth conditions. However, after all, in LAB the glycolytic flow, which in most microorganisms feeds the tricarboxylic acid (TCA) cycle, is redirected in the final phase by converting pyruvate (the end-product of glycolysis) into lactate via a very simple reduction half-reaction ($2C_3H_4O_3$(pyruvic acid) + $4H^+ + 4e^- \leftrightharpoons 2C_3H_6O_3$(lactic acid)) that is carried out by the action of the NAD-dependent enzyme lactate dehydrogenase (LAD)

(Rabinowitz & Enerbäck, 2020). Please note that electrons necessary for the conversion of pyruvate to lactate are provided by two molecules of NADH which are made from NAD^+ at the glyceraldehyde-3-phosphate dehydrogenase step in the middle of glycolysis so that the conversion of one glucose molecule to two lactic acid molecules is electrochemically neutral (i.e., neither produces nor consumes electrons). Therefore, a very intriguing picture is that in the LAB-yeast community the interrupted glycolytic flow in the LAB cells is simply resumed in the yeast cells by reconverting lactic acid to pyruvate ($C_3H_6O_3$(lactic acid) ⇋ $C_3H_4O_3$(pyruvic acid) + $2H^+$ + $2e^-$). In this way the yeast obtains a double energy gain because firstly, a molecule of reduced NADH is made available by the simple oxidation of lactic acid to pyruvate and, secondly, the energy of pyruvate can be fully recovered by conveying it through the yeast TCA cycle and respiratory chain.

Due to its well-known antimicrobial properties, excess lactic acid in single lactic acid bacteria cultures and co-culture is most likely an important factor in determining the antimicrobial properties of CFSs from LAB-containing cultures as set out in Table S2. However, this does not exclude that other antimicrobial agents may play a role in determining the observed antimicrobial activity of CFSs, especially of CFSs from *P. kudriavzevii* individual culture where the level of lactic acid is extremely low.

Since lactic acid bacteria do not oxidize glucose in their energy production, they happily grow under anaerobic conditions, although they can also grow in oxygen's presence. In this regard, we observe that 2,3-butanediol (#1) has been detected only in LAB2 culture and co-culture as we readily deduce from its {1,0,0,4,0} coordinates (see Table S3). From Fig. 5A, we see that 2,3-butanediol has been detected at comparable levels in LAB2 single culture and in the co-culture. We then infer that in the co-culture 2,3-butanediol is synthesized by LAB2 and its production is unaffected by the presence of the yeast. In fact, production of 2,3-butanediol by lactic acid bacteria is well documented. 2,3-Butanediol is biosynthesized from pyruvate via a multistep pathway as an alternative to the reduction of pyruvate to lactic acid (Celińska & Grajek, 2009). Nevertheless, whereas production of lactic acid from glucose is electrochemically neutral, production of 2,3-butanediol is an oxidation of glucose which releases two electrons for each molecule of glucose oxidized ($C_6H_{12}O_6$(glucose) ⇋ $C_4H_{10}O_2$(2,3-butanediol) + $2CO_2$ + $2H^+$ + $2e^-$). So far, the metabolic function of 2,3-butanediol in LAB has not been clarified. However, we suggest that 2,3-butanediol production may play a role in maintaining the cellular NAD^+/NADH balance under conditions of low NADH due, for example, to the presence of oxygen when lactic acid bacteria are grown under aerobic conditions. In fact, one NADH molecule can be regenerated from NAD^+ by employing the two electrons released by the conversion of one glucose molecule to 2,3-butanediol which is useful because NADH is necessary to reduce pyruvate to lactic acid in the last step of the fermentation pathway from glucose to lactic acid.

Even a cursory inspection of Fig. 4 will show that LAB2 and LAB1 have very limited biosynthetic capabilities while *P. kudriavzevii* avidly metabolizes amino acids and other components of the MRS broth and synthesizes a variety of new compounds. For instance, we easily see from Fig. 4 that *P. kudriavzevii* can metabolize trehalose (#9; see Fig. S6) present in the MRS medium since trehalose has coordinates {1,2,0,0,5}. LAB do not seem to appreciate trehalose as an energy source since the level of trehalose in individual cultures of the LAB is the same as in the MRS medium (see Fig. 4A and B). From the trehalose coordinates above we also see that consumption of trehalose by the yeast continues in the co-culture since trehalose is not detected in the co-culture.

Since LAB have limited biosynthetic capacities (Pessione, 2012), they require preformed amino acids, B vitamins, purines, pyrimidines, etc., for survival and growth (Teusink & Molenaar, 2017). In particular, LAB employ a major fraction of the available metabolic energy for the uptake of amino acids from the environment. Since LAB are well adapted to ecological niches where amino acids must be obtained from proteins, they have evolved a complex and efficient proteolytic system (Kieliszek et al., 2021; Pritchard & Coolbear, 1993). We can see the effect of the proteolytic ability of LAB in Fig. 4A and, in a lower grade, in Fig. 4B. By exploring Fig. 4A we can count that in LAB2 individual culture there are 12 proteinogenic amino acids plus ornithine which are upregulated with respect to the MRS broth. This excess amino acid is a suitable ingredient for LAB to produce bacteriocins, which are antimicrobial peptides protecting LAB from life-threatening microorganisms in the environment (Zimina et al., 2020). For instance, it is well known that *L. lactis* subsp. *lactis* synthesizes nisin A, a 34 amino acids oligopeptide with broad-spectrum antimicrobial properties (Hernández-González et al., 2021) and genome mining appears to indicate that the majority of Gram-positive bacteria contain genes encoding these peptides (Alvarez-Sieiro et al., 2016; Zhao & Kuipers, 2016).

Apart from this, we note that UPz metabolites in Fig. 4D have a special significance since they represent metabolites that have been detected in the co-culture and have not been detected in the MRS broth and then, in abstract, are new metabolites produced by the metabolic activity of the microorganisms. Since the UPz metabolites in Fig. 4D will most likely be present in foods fermented by a mixture of the organisms considered, the following discussion is intended to place them in a chemical and biological context.

We first consider a group of alcohols/polyols (i.e., tyrosol (#54), phenylethyl alcohol (#60) and erythritol (#59)) which, as we readily see from their common {0,0,3,4,0} coordinates, are produced by *P. kudriavzevii*. From Fig. 5C we easily see that, compared to the single yeast culture, in the co-culture tyrosol is present at comparable levels, and phenylethyl alcohol and erythritol are downregulated. Tyrosol is found in olive oil and is believed to contribute to the favorable effects of the Mediterranean diet (Marković et al., 2019); phenylethyl alcohol is an antimicrobial, antiseptic, and disinfectant that is used also as an aromatic essence and preservative in pharmaceutics and perfumery (Mitri et al., 2022); erythritol is a sweetener that is almost an ideal substitute for sugar because it has no impact on glycemia and, unlike other polyols, does not have side effects (den Hartog et al., 2010). Thus, the presence of these alcohols/polyols in fermented foods is quite desirable (see Fig. S6). In addition, from Fig. 2 we can see that CFSs from co-cultures turned out to be particularly active in the DPPH free radical test and tyrosol and erythritol could be partly responsible of its antioxidant activity considering the wide range of health-related benefits of tyrosol, including the reduction of oxidative damages (Rodríguez-Morató et al., 2012), and that erythritol is an excellent free radical scavenger (den Hartog et al., 2010).

Erythritol (#59) is the four-carbon polyol (i.e., CH_2OH-CHOH-CHOH-CH_2OH) which has attributes very similar to xylitol (#12), the five-carbon polyol (i.e., CH_2OH-CHOH-CHOH-CHOH-CH_2OH). Erythritol and xylitol are well distinguished by GC–MS because their TMS derivatives have quite different chromatographic characteristics and retention index (see Table 1). From our data, it results that the MRS broth is rich in xylitol which is detected in all cultures except the co-culture as we see from its {1,2,3,0,5} coordinates. This is not surprising since, as we see from Fig. 4A, xylitol is metabolized by LAB2 and is downregulated in class 1LAB2 with respect to 5MRS class (FC = 0.04).

In the co-culture we find tryptophan (#2) and tyrosine (#47) which, interestingly, as we see from their {0,0,0,4,0} coordinates, have not been detected in individual cultures of the microorganisms or in the MRS broth. This is a bad case for our strategy because, apparently, we cannot tell whether these amino acids are excreted into the co-culture by the LAB or by the yeast. However, we note that in the yeast culture we have detected tryptophol (#63) which has not been detected in LAB individual cultures. As the name suggests, tryptophol is biosynthesized from tryptophan. So, phenomenologically, what we understand is that tryptophan is produced by *P. kudriavzevii* in both the individual culture and the co-culture, but while in the individual yeast culture tryptophan is converted to tryptophol, in the co-culture it is excreted and made available to the lactic acid bacteria. This is a beneficial effect of LAB on yeast metabolism, since tryptophol is known to induce sleep in humans

and, in general, its presence in foods is questionable, while the presence of tryptophan is very welcome because tryptophan is an essential amino acid for humans. Similarly to tryptophan, we can infer that tyrosine is very likely excreted in co-culture by *P. kudriavzevii* since tyrosine is the precursor of tyrosol which, as we showed above, is synthesized by the yeast.

Trp and Tyr secretion by *P. kudriavzevii* in co-culture is reminiscent of the nitrogen overflow effect which is observed when *Saccharomyces cerevisiae* is co-cultured with *Lactobacillus plantarum* and *Lactococcus lactis*. Overflow occurs in nitrogen rich communities where *Saccharomyces cerevisiae* changes its metabolism to secrete pools of metabolites such as amino acids which benefits lactic acid bacteria (Ponomarova et al., 2017).

Ornithine (#44) is an UPz metabolite in Fig. 4D which is found in all cultures tested either as such or as cycloornithine (#38). It is inferred that LAB2, LAB1 and *P. kudriavzevii* have the ability to synthesize ornithine which can be derived from glutamic acid (#43, {1,2,0,4,5}) present in the MRS broth (Wu et al., 2020) and avidly metabolized by the yeast (see Fig. S6). Ornithine is the precursor of putrescine (#50) a biogenic amine which can be derived from ornithine by a simple decarboxylation reaction (i.e., ornithine ⇋ putrescine + CO_2). From our data, it appears that only *P. kudriavzevii* is capable of converting ornithine to putrescine ({0,0,3,4,0}), since we did not detect putrescine in single cultures of LAB2 and LAB1. Furthermore, we find that the level of putrescine in the co-culture is comparable to its level in the *P. kudriavzevii* individual culture (see Fig. 5C). Like putrescine, its biogenic amine homologue, cadaverine (#49; see Fig. S6), is obtained by decarboxylation of lysine (#10) but, although lysine ({1,2,3,4,0}) is present in all cultures analyzed, cadaverine ({0,0,3,4,0}) is, as observed for putrescine, only detected in the *P. kudriavzevii* culture and in the co-culture (see Fig. 5C). However, unlike putrescine, production of cadaverine by the yeast is stimulated by the presence of LAB (see Fig. 5C) since it is upregulated in class 4CoCulture with respect to class 3PICHIA (FC = 12.8). Despite their unpleasant odor, cadaverine and putrescine are among the most important metabolites produced by intestinal microbiota affecting the health and disease of the host due to their multiple physiological roles. In fact, these polyamines have many functions, such as the maturation and maintenance of intestinal mucosal barrier, anti-inflammatory actions, antimutagenicity, and autophagy (Matsumoto et al., 2012). Thus, their oral administration via fortified foods with probiotic and/or postbiotic preparations could have a positive role in the maintenance of a favorable host-microbial association.

As said above, LAB2 and LAB1 synthesize ornithine but they do not use it in the way *P. kudriavzevii* does. From our data, we can infer that LAB2 and LAB1 employ ornithine to synthesize proline (#40). Indeed, as we can see by exploring Fig. 4, proline ({1,2,0,4,0}) is not detected either in the MRS medium or in the *P. kudriavzevii* culture but is detected in LAB2, LAB1 cultures and in the co-culture is upregulated with respect to the bacterial cultures (see Fig. 5A and B). The conversion of ornithine to proline is chemically quite simple and may consist in the reaction: Ornithine ⇋ Proline + NH_3 (see Fig. S6). However, this reaction requires the enzyme ornithine cyclodeaminase (OCD) which so far has been demonstrated only in a few organisms such as *Clostridium sporogenes*, *Treponema denticola*, *Agrobacterium tumefaciens*, and *Pseudomonas putida* (Jensen & Wendisch, 2013). In addition to be a major constituent of proteins and bacteriocins, proline also acts as an osmotic protectant in bacteria, plants and animals that are under osmotic stress, and it also results to be a potent ROS scavenger in particular towards singlet oxygen (Michaletti et al., 2018). Accumulation of proline in co-culture may then be a factor helping lactic acid bacteria to reduce stress induced by the presence of the yeast. Furthermore, from our data we find that LAB2 also employs proline to produce the dipeptide Pro-Ala (#3; {1,0,0,4,0}) which is a secondary metabolite exclusively detected both in the LAB2 culture and in the co-culture. It seems that LAB2 bacterium maximizes the amount of proline in the co-culture simply by decreasing production of Pro-Ala dipeptide which, as can be seen in Fig. 5A, is downregulated in the co-culture with respect to LAB2 individual culture (FC = 0.19). Since alanine (#39) is required in addition to proline to synthesize the dipeptide Pro-Ala, we expect an upregulation of alanine in both the LAB2 culture and the co-culture. Fig. 4A and D confirm this expectation.

Among the UPz metabolites in Fig. 4D, we find several alpha hydroxycarboxylic acids. In particular, 2-hydroxyisocaproic acid (#41; see Fig. S6)) was detected in all cultures analyzed as we see from its {1,2,3,4,0} coordinates. In fact, production of 2-hydroxyisocaproic acid by *Leuconostoc lactis*, a lactic acid bacterium isolated from kimchi, has already been reported (Park et al., 2017), but from our data we see that *P. kudriavzevii* can also synthesize this metabolite. However, it seems that in the co-culture this alpha hydroxy acid is mainly produced by LAB2 since its level in the CFS from co-culture is similar to the level in the CFS from LAB2 culture (see Fig. 5A) and it is significantly higher than the level in CFSs from individual cultures of LAB1 and *P. kudriavzevii* (see Fig. 5B and C). 2-Hydroxyisocaproic acid has mild fungicidal or antibiotic activities against *Candida*, *Aspergillus*, *Staphylococcus*, and *Fusobacterium* and also against *Enterococcus faecalis* isolated from human teeth (Pahalagedara et al., 2022; Park et al., 2017). This suggests its possible involvement in the observed antimicrobial and antibiofilm activities of CFSs against *Salmonella* sp. S1 (see Table S2, Fig. 1 and Fig. S1).

2-Hydroxyisocaproic is also known as leucic acid because it is biosynthesized from leucine (#34). The biosynthesis of 2-hydroxyisocaproic acid ($C_6H_{12}O_3$) begins with a transamination reaction which converts leucine ($C_6H_{13}NO_2$) to 2-ketoisocaproic acid ($C_6H_{10}O_3$) which is then reduced to 2-hydroxyisocaproic acid. It is evident that it is possible to create an alpha hydroxy acid by subjecting any alpha amino acid to the sequence of reactions which converts leucine to 2-hydroxyisocaproic acid. For instance, applying this reaction scheme to phenylalanine (#33), we obtain phenyllactic acid (#53). However, although it is reported that many strains of lactic acid bacteria can produce phenyllactic acid (Mu et al., 2009) and phenylalanine ({1,2,0,4,5}) is present in the MRS broth and in CFSs from the LAB cultures, we have not detected phenyllactic acid in the individual cultures of the lactic acid bacteria. In fact, as we see from its {0,0,3,4,0} coordinates, phenyllactic acid is detected only in the yeast culture and in the co-culture at levels not significantly different (see Fig. 5C). From this we deduce that only *P. kudriavzevii* is capable of converting phenylalanine to phenyllactic acid and its production is unrelated to presence of LAB. Since phenylalanine is required for the biosynthesis of phenyllactic acid, this view is consistent with the fact that *P. kudriavzevii* avidly metabolizes phenylalanine from the MRS medium, while the level of this amino acid is equal to or even higher than that in the MRS medium in LAB1 and LAB2 cultures, respectively (see Fig. 4). Phenyllactic acid has been shown to possess a broad-spectrum antimicrobial and antibiofilm activities against an assortment of bacteria, fungi and yeasts and has therefore attracted much interest as a preservative for foods, cosmetics, and pharmaceuticals (Jiang et al., 2022; Maione et al., 2023). Consequently, the presence of phenyllactic acid as an endogenous preservative in fermented foods would be welcome. Finally, similarly to phenyllactic acid, 2-hydroxy-2-methylbutyric acid (#55, {0,0,3,4,0}) is also produced by *P. kudriavzevii* and is detected exclusively in the yeast culture and in the co-culture at not significantly different levels (although for this alpha hydroxy acid does not exist a proteinogenic amino acid from which it can be derived by applying the above reaction scheme).

In summary, this study unraveled several interesting metabolites and complementary metabolic patterns in the *L. lactis* subsp. *lactis* LAB2, *L. lactis* subsp. *hordniae* LAB1 and *P. kudriavzevii* Y1 community which can be of value to explain the beneficial effects of multiprobiotic LAB/yeasts preparations and contributes concepts and knowledge for their application in the fermented food industry. Meanwhile, we developed a general workflow for acquiring and evaluating GC–MS metabolomic data which can be extrapolated to identify and disentangle metabolic interactions between microorganisms in co-cultures.

Funding

This research was supported by funding from PRIN2022— Financed by European Union—Next Generation EU. Serum metabolomics in atopic dermatitis (MetabAD): the potential of a GC–MS based strategy to improve the clinical diagnosis, the understanding of the disease, and the treatment efficacy (No. 2022HC3FRM).

CRediT authorship contribution statement

Maria Michela Salvatore: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angela Maione:** Visualization, Investigation, Formal analysis, Data curation. **Annalisa Buonanno:** Investigation. **Marco Guida:** Supervision, Resources. **Anna Andolfi:** Supervision, Conceptualization. **Francesco Salvatore:** Writing – review & editing, Supervision, Software. **Emilia Galdiero:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142657.

Data availability

Data will be made available on request.

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