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Effect of sequential or ternary starters-assisted fermentation on the phenolic and glucosinolate profiles of sauerkraut in comparison with spontaneous fermentation

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Abstract:	This study investigated the effectiveness of two novel started-assisted sauerkraut fermentations in comparison with spontaneous fermentation. Three lactic acid bacteria strains were selected as best starters for sauerkraut processing, based on the complementarity of pro-technological (kinetics of growth and acidification) traits, phenotypic fingerprints through OmniLog Phenotype MicroArray, and phenolics metabolism. The selected strains were applied according to two different fermentation methods based on steering sequential and temporally deferred inoculum of three strains, and ternary simultaneous inoculum. Sequential and ternary starters-assisted fermentation lasted 9 and 7 days, respectively, and were compared to conventional spontaneous fermentation lasting 35 days. Sequential and ternary fermentation resulted in a higher and constant number of lactic acid bacteria compared to spontaneous fermentation, which reflected on the acidification and sugar utilization. Ternary fermentation enhanced phenolic compounds conversion (hydrocaffeic acid, hydroferulic acid, 4-ethyl catechol), ensuring at the same time higher level of aliphatic (glucobrassicanapin) and indole glucosinolates (glucobrassicin) and derivatives (ascorbigen). Short fermentation of sequential and ternary starters-assisted processing caused only slight changes in the sensory profile compared to the spontaneous process, preserved the structural integrity of sauerkraut, and did not affect the colour lightness.

Bari, 4th March 2022

Dear Editor,

I would like to thank you and the referee for giving us the opportunity to improve the manuscript. Please, note that all the recommendations, none excluded, have been considered in the revised version. An itemized list of the revisions according to the referee's recommendations has been provided.

Kind regards,

Pasquale Filannino.

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I would like to thank you and the referee for giving us the opportunity to improve the manuscript. Please, note that all the recommendations, none excluded, have been considered in the revised version. An itemized list of the revisions according to the referee's recommendations has been provided.

Kind regards,

Pasquale Filannino.

Reviewer reports:

Reviewer #1

Reviewer #1: This study compared traditional and two new fermentation methods to produce sauerkraut, and analyzed parameters including the microbes, the metabolism chemicals and phenotypic fingerprints. The results provide references for rapid and high valued sauerkraut fermentation.

What is the scale of the fermentation? Sauerkraut fermentation was carried out in laboratory scale. A clarificatory sentence has been added (P8 L28).

Reviewer #2

Reviewer #2: Congratulations for your excellent work. I have a made a few notes directly in the text.

All the keywords are already on the title, you may consider choosing some other keywords to enhance the article visibility. **Ok**, new keywords have been provided, also taking into account the comment of reviewer 4 (P2 L48-50).

Consider breaking the paragraph when changing the topic of what is being discussed. The text is well written, but long paragraphs makes it exhausting to read. **Ok**, the paragraphs have been appropriately subdivided according to your suggestions (P3 L28; P14 L41; P15 L46).

Is this the correct structure? **Ok**, the sentence has been rephrased more appropriately (P3 L52).

Revise the titles of the material and methods section - f, k and h are in a different format. **Ok**, the format has been standardized throughout the manuscript.

Check if the color system used was L a b or L* a* b*. If it was the latter, please correct the manuscript. **Ok**, ok the right system (L* a* b*) has been reported throughout the manuscript. We apologize for the imprecision.

Please, explain the difference in the sampling times from the different fermentation treatments. **Ok**, A clarificatory sentence has been added (P9 L198-200). Sampling times were chosen to ensure wide coverage of fermentation processes, which varied in duration, and taking into account the pH shifts.

I suggest writing the extended version again (glucosinolates) and use the abbreviation into brackets (GLSs). **Ok**, the extended version was reported according to your suggestions (P11 L42; P20 L52).

Why? Assuming that each cluster groups strains with similar behaviour, for each cluster we selected one representative strain to be further characterization. The sentence has been revised within the manuscript to further clarify this aspect (P16 L11).

Consider rewriting for better understanding of the progression of the bacterial growth. **Ok**, the sentence has been rephrased (P17 L50-52; P18 L11-18).

Figure 3. Consider changing the color of the strain S2D5, as ir it very similar to the color used for the C source labels, making it difficult to find it on the graph. **Ok**, the colour of C source labels has been changed according to your suggestion, so as not to create any confusion (please, see the new Figure 3).

Graphical abstract. Consider changing Inoculated by Inoculation to match the verbe tense used in the rest of the graphic. **Ok**, “inoculated” was changed by “inoculation”.

Overall, the manuscript is well written and of good scientific quality.

Reviewer #3

Reviewer #3: The manuscript "Sequential or ternary starters-assisted sauerkraut fermentation affect the profiles of phenolics and glucosinolates differently" has important information about the influence of the starter culture during the fermentation process of sauerkraut. However, to be published in Food Research International the article needs to be improved.

Abstract: the authors could add more data. **Ok**, we detailed the abstract with more information, also taking into account the comments of other referees.

Line 148: Spontaneous fermentation of sauerkraut occur by 36 days. Why the author evaluate phenolic compound only with 24h of fermentation? **Ok**, an explanation has been also added within the manuscript (P7 L20-24). You are right, the fermentation of sauerkraut is longer than 24 h, but the assay in object is an exploratory screening to verify the enzymatic equipment of each strain. The growth conditions of the assay are different from those of the actual sauerkraut fermentation, as the microorganism is grown in purity, is inoculated at high cell density, and the plant molecules are readily available in the juice. This allows the metabolic performance of each strain to be verified more quickly. The same approach was previously adopted by other authors (Xu et al., 2021).

Line 179: Is there some reason to sequential inoculation? Explain. **Ok**, an explanation has been also added within the manuscript (P8 L46-50). The order of inoculation of each strain reproduced the appearance and dominance of the different species of lactic acid bacteria during spontaneous fermentation of the sauerkraut, as previously investigated by Lemos Junior et al. (2022). The time of inoculum for each species was based on preliminary trials where pH, cell density of lactic acid bacteria, and organic acids determined periodically defined the time interval between each inoculation.

Line 180: Rewritten the sentence. Detail the methodology with data detected by Tlais et al., 2021b. **Ok**, the sentence has been rephrased and additional information was added (P8 L50-52; P9 L11-13).

Tlais et al., 2021b: This paper has not yet been accepted for publication. Consider replacing or adding data. **Ok**, the reference was revised as follow: Lemos Junior W. J. F., Tlais, A. Z. A., Filannino, P., Campanaro, S., Gobbetti, M., Di Cagno, R. (2022). How microbiome composition correlates with biochemical changes during sauerkraut fermentation: a focus on neglected bacterial players and functionalities. Microbiology Spectrum. (under review)

Line 203: Add the mobile phase and temperature of analyses. **Ok**, information has been added (P10 L29-31; P10 L35-37). For sugar analysis, elution was at 32 °C, with a flow rate of 1 ml min^{-1}, using acetonitrile 80% as mobile phase. For organic acids analysis, elution was at 70 °C, with a flow rate of 0.6 ml min^{-1}, using H_2SO_4 5 mM as mobile phase.

Line 278: Add the sensory attribute. **Ok**, sensory attributes are reported at P13 L40-43.

Line 352 and 355: Exemplify carbon sources. Details of the major sources consumed are described for each strain at P16 L29-31, P16 L31-33, P16 L33-37.

Line 382: What are the contributions of yeasts to the sauerkraut fermentation process? Are they essential? If not, the authors should explore this result further. **Ok**, the discussion of the role of yeasts has been included in the manuscript (P18 L24-35). Yeasts are considered undesirable microorganisms, especially in the late stages of sauerkraut fermentation and during storage (Yang et al., 2020). They are high producers of pectinase which might be responsible for the softening and deterioration of fermented vegetables (Pino et al. 2019). Yeasts are usually present during the early phase of the sauerkraut fermentation and their growth is highly dependent on the salt concentration in brine, as well as the use of the starter culture (Müller et al., 2018), which might explain their absence in Seq and Ter fermented sauerkrauts.

The article's discussion is succinct and needs improvement. The authors need to describe more about the metabolism of lactic acid bacteria and their interactions. Further, show how relevant starter culture are to the fermentation process. **Ok**, the metabolism of bacteria, microbial interactions and the importance of starters have been discussed more in detail throughout the manuscript.

Why ternary fermentation show a better methodology of inoculation? Is processing time an important parameter? If yes, emphasize the result. **Ok**, we made the meaning of our results more explicit within the manuscript. Our results clearly indicated that the timing of inoculation had a significant effect on the profiles of GLS, as well as phenolics, and can be used to manipulate the profile of bioactive compounds in sauerkraut and to develop ad-hoc fermented products for health promoting purpose.

Scientific names need to be revised. **Ok**, scientific names have been revised throughout the manuscript.

The picture quality needs to be improved. **Ok**, the resolution of all figures has been strongly improved.

Table 1: The statistical data is not clear. Fix the column titles. **Ok**, the meaning of the statistic has been clarified and the column titles have been fixed. Please, see new Table 1.

I suggest replacing the PCA with a table containing statistical data. **Ok**, we kept the PCA graph, but we added the Tables S3 and S4 with statistical data.

Reviewer #4

Reviewer #4: The manuscript, entitled "Sequential or ternary starters-assisted sauerkraut fermentation affect the profiles of phenolics and glucosinolates differently" (ID: FOODRES-D-21-04435), by Ali Zein Alabiden Tlais and other co-authors investigated the effect of sequential (Seq) or ternary (Ter) methods on the phenolic and glucosinolate composition of sauerkraut. Ternary method was found to be more effective for the phenolic compound conversion and also for higher levels of glucosinolate derivatives. Some of the results in this study are interesting and valuable to the related research area. However, in my opinion, the manuscript needs major revisions before it can be accepted for publication in Food Research International. The following suggestions are provided to only help the authors improve on the currently submitted version of the manuscript. **Ok**, we overall improved the manuscript, also thanks to your comments and those of the other reviewers.

1. Title: The current version of title is suggested to be revised as "Effect of sequential or ternary starters-assisted fermentations on the phenolic and glucosinolate profiles of sauerkraut". **Ok**, the title has been modified in accordance with your suggestion and subsequent comments.

2. The abbreviations of ternary (Ter), sequential (Seq) and spontaneous (Sp) are suggested to be deleted in the Abstract. All of them can be replaced by their corresponding full names. **Ok**, abbreviations have been replaced by the extended words.

Another concern about the experimental design is that actually three fermentation approaches including ternary (Ter), sequential (Seq) and spontaneous (Sp) were investigated and compared in this study. Why only ternary (Ter) and sequential (Seq) mentioned in the Title and at the beginning of the Abstract. Thank you for your suggestion. The survey of spontaneous fermentation has been highlighted in both the title and the abstract.

3. As the research focus of the present study, "ternary" and "sequential" methods should be included in the Keyword list. **Ok**, they were added as new keywords, which were modified also taking into account the comment of reviewer 2 (P2 L48-50).

4. Line 48: What did "the first processed products" refer to? **Ok**, the meaning has been clarified within the manuscript (P3 L13). Fermented foods and beverages are among the most ancient processed products consumed by humans.

5. Lines 52-56: The introduction concerning the nutrients and the functional metabolites should be given to the fermented cabbage or sauerkraut, not the fresh cabbage. Were there any references or publications available on the chemical or nutrient composition of sauerkraut?? If yes, the related information should be reviewed and cited in the Introduction. **Ok**, the focus has been shifted to the effects of fermentation on nutritional and functional value of sauerkraut, with relevant literature references.

6. Lines 78-79: In addition to Ray & Bhunia (2007), more recently published and related papers should be cited here. **Ok**, new latest references have been added (Yang et al., 2020; Hu, Yang, Ji, & Guan, 2021).

7. 2.2.3. Free phenolic compounds metabolism For the analysis of free phenolic compounds, what types of platforms were used, targeted or non-targeted? If the targeted method used, how many phenolic compounds were analyzed, and the corresponding authentic standard information should be provided. On the other hand, if the non-targeted one applied, more detailed method information should be provided. Why different methods were used for the analysis of free phenolic compounds in fermented cabbage juices and in sauerkraut (Section 2.6)? A targeted metabolomics method has been used for the identification and quantification of 24 phenolics in sauerkraut by using LC-ESI-MS/MS. Target phenolics were detectable under multiple reaction monitoring (MRM) mode and the compounds were identified based on their reference standard, retention time, qualifier and quantifier ion (please, see new Table S2). More details have been added within the manuscript (P11 L16-18, P11 L26-30). A non-targeted metabolomics method was used for the analysis of cabbage juice as preliminary screening method to capture all the differences among the strains

in term of metabolic performances (thanks to a comprehensive look at all phenolic compounds, not only the targeted ones).

8. Abbreviations such as GLSs should be avoided in the subtitles. Please check through the manuscript and make revisions accordingly. **Ok**, the extended version was reported according to your suggestions and that of reviewer 2 (P11 L42; P20 L52).

9. Why the information and the results of color, texture and sensory analysis of sauerkraut was not mentioned and presented in the title, highlights and abstract of the manuscript? In my opinion, these data are also very important for the quality evaluation of sauerkraut fermented by different methods. **Ok**, you are right. The impact of the three fermentation methods on the organoleptic profile has been better highlighted in both the abstract and the conclusions sections.

10. The authors are suggested to point out the best or the most optimal fermentation method (ternary or sequential) for sauerkraut in the Conclusion. **Ok**, the best method has been highlighted (P24 L44-50). Ternary cultures can be used to manipulate the profile of bioactive compounds in sauerkraut in order to develop ad-hoc fermented products for health promoting purpose, limiting at the same time the negative consequences of a short fermentation on the typical organoleptic profile of sauerkraut.

11. The references should be revised to ensure that all of them were presented in a consistent format. **Ok**, the format has been carefully revised.

12. The format of figures should be revised to improve the resolution. The current version is not clear for read. **Ok**, the resolution of all figures has been strongly improved.

Highlights

- Two sauerkraut fermentation methods based on selected starters were investigated
- Strains were inoculated according on sequential (Seq) or ternary (Ter) methods
- Seq and Ter methods showed higher acidification rate than spontaneous fermentation
- Ter fermentation enhanced phenolics conversion
- Ter method led to higher level of glucobrassicanapin, glucobrassicin, ascorbigen

Effect of sequential or ternary starters-assisted fermentation on the phenolic and glucosinolate profiles of sauerkraut in comparison with spontaneous fermentation

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Abstract

This study investigated the effectiveness of two novel started-assisted sauerkraut fermentations in comparison with spontaneous fermentation. Three lactic acid bacteria strains were selected as best starters for sauerkraut processing, based on the complementarity of pro-technological (kinetics of growth and acidification) traits, phenotypic fingerprints through OmniLog Phenotype MicroArray, and phenolics metabolism.

The selected strains were applied according to two different fermentation methods based on steering sequential and temporally deferred inoculum of three strains, and ternary simultaneous inoculum. Sequential and ternary starters-assisted fermentation lasted 9 and 7 days, respectively, and were compared to conventional spontaneous fermentation lasting 35 days. Sequential and ternary fermentation resulted in a higher and constant number of lactic acid bacteria compared to spontaneous fermentation, which reflected on the acidification and sugar utilization. Ternary fermentation enhanced phenolic compounds conversion (hydrocaffeic acid, hydroferulic acid, 4-ethyl catechol), ensuring at the same time higher level of aliphatic (glucobrassicanapin) and indole glucosinolates (glucobrassicin) and derivatives (ascorbigen). Short fermentation of sequential and ternary starters-assisted processing caused only slight changes in the sensory profile compared to the spontaneous process, preserved the structural integrity of sauerkraut, and did not affect the colour lightness.

Keywords: Sauerkraut ~~fermentation~~, ~~Starter cultures, Phenolic compounds, Glucosinolates~~controlled fermentation, ternary starter, sequential inoculum, bioactive compounds.

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1. Introduction

Fermented foods and beverages are among the most ancient processed products consumed by humans, are staples in the human diet, and, recently, have been included as part of dietary recommendations (Marco et al., 2017). Among fermented vegetables, sauerkraut represents one of the most common and oldest forms of preserved cabbage (*Brassica oleracea* convar. *capitata* var. *sabauda* L) or pointed cabbage (*Brassica oleracea* var. *capitata* f. alba) (Martinez-Villalueng et al., 2009). Upon fermentation, the chemical composition of cabbage changes, resulting in a product rich in functional metabolites and nutrients, among which phenolic and glucosinolate (GLS) breakdown products attracted attention for their health modulating properties (Martinez-Villaluenga et al., 2009; Peñas, Martinez-Villaluenga, & Frias, 2017).

Traditionally the production of sauerkraut occurs through spontaneous lactic acid fermentation Hence, the quality of sauerkraut depends on the naturally occurring lactic acid bacteria, their succession, and their metabolic activities. As drawbacks, spontaneous fermentation takes a long time, which may cause unpredictable and unstandardized sensory and organoleptic properties, as well as the risk of the occurrence of spoiling and pathogen bacteria, and detrimental biogenic and polyamines (Peñas, Frias, Sidro, & Vidal-Valverde, 2010). Consequently, the use of starter cultures for fermented vegetables has recently been encouraged (Torres, Verón, Contreras, & Isla, 2020). The selection of allochthonous or autochthonous starters is a critical procedure for sauerkraut fermentation, which have to reflect the typical succession of lactic acid bacteria species, and to ensure safety and improve the overall quality (De Melo Pereira et al., 2019). Selection should be based mainly on environmental adaptation, which affect all the other potential metabolic traits and the persistence throughout fermentation (Di Cagno, Coda, De Angelis, & Gobbetti, 2013; Filannino, Di Cagno, & Gobbetti, 2018). ~~The equipment of a~~An optimal portfolio of enzymes ~~and~~

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~~metabolic traits~~ enables starters to follow successfully different metabolic pathways. Complementary enzymes having as target polyphenols and glucosinolates such us glycosyl hydrolases, esterases, decarboxylase, and reductase provides the capability to metabolize them into several derivatives during the fermentation, which may exert higher biological activities than the precursors (Palani et al., 2016; Narbad & Rossiter, 2018; Tlais, Fiorino, Polo, Filannino, & Di Cagno, 2020). Earlier studies revealed the efficiency of lactic acid bacterial starters to ferment cabbage and other vegetables (Ray & Bhunia, 2007; Yang et al., 2020; Hu, Yang, Ji, & Guan, 2021). All agreed that the use of suitable starter(s) resulted in accelerated fermentation time compared to spontaneous fermentation. Anyway, studies aimed to select starters for sauerkraut fermentation were only dedicated to the investigation of single starter, condition quite far from that of spontaneous fermentation, and were mainly focused on growth rate, acidification capacity, nitrite depletion ability, and antimicrobial activity (Martinez-Villaluenga et al., 2009; Yang et al., 2020). When the fermented food is subjected to a long fermentation process reflecting a distinctive microbial succession over time, as in the case of sauerkraut, it is difficult and restrictive to propose a single starter. The application of selected mixed cultures rather than a single strain might be more worthwhile to carry out successful tailored fermentation of sauerkraut. However, the management of mixed cultures remains difficult since it requires an optimum balance and the right succession of selected lactic acid bacteria strains.

In this study, we proposed two fermentation protocols to overcome the drawback associated to a long fermentation, and at the same time to obtain sauerkraut enriched in bioactive compounds. Among lactic acid bacteria strains, previously isolated from spontaneous fermented cabbage, we selected the best performing ones according to their kinetics of growth and acidification, phenotypic fingerprints, and phenolics metabolism. The selected strains were applied according to

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two different methods based on steering sequential (inoculum of three lactic acid bacteria species temporally deferred) and ternary (simultaneous inoculum of three lactic acid bacteria species) fermentation. A comparison was carried out with the traditional spontaneous method. How the sequential, ternary, and spontaneous sauerkraut fermentations at pilot level affected the metabolism of phenolic compounds and glucosinolates, fermentation effectiveness, sensory and texture properties of sauerkraut were investigated in this study.

2. Materials and methods

2.1. Microorganisms and culture conditions

Twenty-three strains of lactic acid bacteria were used in this study (Table S1): *Leuconostoc citreum* (7 strains), *Leuconostoc suionicum* (1), *Leuconostoc mesenteroides* (4), *Lacticaseibacillus paracasei* (1), *Pediococcus parvulus* (9), *Lactiplantibacillus plantarum* (1). All strains belonged to the Culture Collection of the Micro4Food, Free University of Bolzano, Bolzano, Italy, and were previously isolated during spontaneous fermentation of sauerkraut. Strains were identified by partial sequencing of the 16S rRNA (Table S1). Cultures were maintained as stocks in 15% (vol/vol) glycerol at −80 °C, and routinely propagated at 30°C for 24 h in MRS broth (Oxoid, Basingstoke, Hampshire, United Kingdom).

2.2 Starter selection

2.2.1. Cabbage juice preparation

Cabbage juice was used as the growth model system to investigate the kinetics of growth and acidification and metabolism of phenolic compounds by lactic acid bacteria strains. Although cabbage juice represents a simplified growth model compared to raw cabbage, compounds of interest from microbiological, technological, and nutritional perspectives are present in cabbage

juice in appreciable amounts, and its use as a growth medium is supported by previous studies. (Hallmann et al., 2017; Ciska, Honke, & Drabińska, 2021; Xu et al., 2021). Juice medium was prepared as described by Filannino et al. (2020) with a few modifications. White cabbages cultivated in South Tyrol were purchased from local supermarkets. They were chopped into small pieces, 1.4% of salt (NaCl) was added and blended in distilled water 50% (wt/vol) (Classic Blender 400, PBI International). The mixture was shaken for 1 h at room temperature, centrifuged (12500 rpm, 20 min at 4°C), and sterilized by filtration through 0.22 µm membrane filters (Millipore Corporation, Bedford, MA). Once prepared, cabbage juice was kept at −20°C until use.

2.2.2. Kinetics of growth and acidification

Lactic acid bacteria strains were propagated in MRS broth at 30°C for 16-20 h, until the late exponential (LE) growth phase was reached. Then, cells were harvested by centrifugation (10,000 rpm, 10 min at 4°C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), and singly inoculated in cabbage juice to a final cell density of ca. 7.0 log CFU/ml. Cabbage juice was incubated at 30°C and the kinetics of growth and acidification were determined. Growth was monitored by measuring the optical density at 620 nm. The pH was measured by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). Kinetics of growth and acidification were determined and modeled according to the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, & Van't Riet (1990): $y=k + A$ or $\Delta pH \exp\{-\exp[(\mu_{max}$ or V_{max} e/A or $\Delta pH)(\lambda - t) + 1]\}$. When the optical density (OD_{620}) was the dependent variable to be modelled, k was the initial level of optical density (OD_{620} units), A was the difference in OD_{620} units between inoculation and the stationary phase, μ_{max} was the maximum growth rate (OD_{620} units h^{-1}), λ was the length of the lag phase (hours), and t was the time (hours). When the pH (pH units) was the dependent variable to be modelled, k was the initial level of pH, ΔpH was the difference in pH units between inoculation

and the stationary phase, V_{max} was the maximum acidification rate (pH h^{-1}), λ was the length of the lag phase (hours), and t was the time (hours). Data were fitted using non-linear regression procedure of the Statistica 7.0 software (Statsoft, Tulsa, USA).

2.2.3. Free phenolic compounds metabolism

An explorative screening for the ability to metabolize free phenolics was carried through HPLC analyses according to the method described by Tlais et al. (2021a). The assay conditions allowed the metabolic performance of each strain to be assessed during shorter incubations compared to the length of sauerkraut fermentation (Xu et al., 2021). Briefly, free phenolic compounds in fermented cabbage juices were identified and quantified after 24 h of fermentation through an HPLC system Ultimate 3000 (Dionex, Germering, Germany) equipped with photodiode array detector (PAD 3000), low-pressure pump Ultimate 3000, injector loop Rheodyne (Rheodyne, USA, volume 20 µL), and Kinetex C18 Phenomenex (150 mm×4.6 mm with a particle size of 5 µm) column (Thermo Fisher Scientific). Chromeleon Software version 7 (Dionex, Germering, Germany) was used for instrument control, data acquisition and data analysis.

2.2.4. Phenotypic microarray analysis

Lactic acid bacteria strains showing the greatest capacities of growth, acidification and phenolic compounds metabolism were characterized for their carbon consumption profile using OmniLog Phenotype MicroArray (PM) Technology (Biolog, Inc., Hayward, CA, USA). PM plates (Biolog) containing 190 carbon sources (PM1 and PM2) were used. Phenotypic microarray analyses were performed with two biological replicates for each growth condition in accordance with the manufacturer's instructions. Cells were collected when the late exponential (LE) growth phase was reached (ca. 18 h) on cabbage juice medium. Then, cells were washed in 50 mM sterile potassium phosphate buffer (pH 7.0) and diluted (to achieve 65% transmittance) in inoculating

fluid (Biolog) to inoculate the PM plates. One hundred µl of cell suspension were added per each well. Plates were incubated for 48 h (PM1 and PM2). During incubation, reduction of tetrazolium dye by respiring cells was measured in each well every 15 min by the OmniLog system. Generated longitudinal data were analyzed using Micro4Food PM pipeline (Acin-Albiac, Filannino, Gobbetti, & Di Cagno, 2020). Briefly, metabolic profiles were categorized as active and non-active. Metabolic signals were normalized per replicate and array. After removal of common non-active profiles (threshold = 50), metabolic parameters were computed using a free-spline method and confidence intervals (CI) were determined through bootstrapping (n=100).

2.3. Sauerkraut fermentation

Sauerkraut fermentation was carried out in laboratory scale. Cabbages were washed, dried, chopped into small pieces and put in a jar containing 1.4% of NaCl (wt/vol). Pieces were completely submerged underneath the juice and brine released by squeezing out the small pieces. Subsequently, *Leuconostoc mesenteroides* S3d1, *Pediococcus parvulus* S2w2 and *Lactiplantibacillus plantarum* S6w5, selected as starters, were added at an initial cell density of ca. 7 Log CFU/g. For each method of fermentation (Figure 1) three replicates were prepared. One method included the spontaneous fermentation without microbial inoculum (Sp). The sequential fermentation (Seq) included the inoculum of three selected lactic acid bacteria species temporally deferred. *Leuc. mesenteroides* S3d1 was inoculated at D0 (0 day), *P. parvulus* S2w2 at D1 (after 1 day of fermentation) and *L. plantarum* S6w5 at D4 (after 4 days). The order of inoculation of each strain reproduced the appearance and dominance of the different species of lactic acid bacteria during spontaneous fermentation of the sauerkraut, as previously investigated (Lemos Junior et al., 2022). According to Lemos Junior et al. (2022), *Leuconostoc* spp. were dominant during the first days of fermentation, subsequently *P. parvulus* prevailed between 14 and 35 days, and at the end

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of fermentation sauerkraut was mainly dominated by *L. plantarum*. In the present study, the time of inoculum for each species was based on preliminary trials where pH, cell density of lactic acid bacteria, and organic acids levels determined the time interval between each inoculation (data not shown). The third method was the ternary fermentation (Ter) through the simultaneous inoculum of the three selected lactic acid bacteria species (Figure 1). The spontaneous fermentation was carried out a 15°C for 35 days according to the traditional procedure (Thakur, Panja, Das, & Kabir, 2017). Seq and Ter fermentations were at the same temperature and lasted 9 and 7 days, respectively. Each lasting of fermentation was defined based on target values of pH, organic acids and bacteria cell density characterizing spontaneously fermented sauerkraut (Thakur et al., 2017; Lemos Junior et al., 2022). Values of pH, organic acids, carbohydrates, and microbiological analysis were determined as described below on brine and sauerkraut. Samples were taken at the beginning of fermentation (D0), after 2 (D2), 4 (D4), 7 (D7), 9 (D9), 14 (D14), 21 (D21), 28 (D28) and 35 (D35) days of fermentation for Sp, after 1 (D1), 4 (D4), and 9 (D9) days for Sq, and after 1 (D1), 3 (D3), 5 (D5) and 7 (D7) days for Ter. Sampling times were chosen to ensure wide coverage of fermentation processes, which varied in duration, and taking into account the pH shifts.

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2.4. Microbiological and pH analysis of sauerkraut

Samples of sauerkrauts (10 g) were suspended into sterile 0.9% (wt/vol) NaCl and homogenized using a Stomacher apparatus (Seward, London, UK) for 3 min at room temperature. Mesophilic lactic acid bacteria and yeasts were determined on MRS agar (Oxoid) containing 0.1% of cycloheximide (Sigma Cemical Co.) at 30°C for 48 and 72 h under anaerobiosis and on Yeast extract-Peptone-Dextrose agar (YPD, Oxoid), added of 150 ppm chloramphenicol at 30°C for 72

h, respectively. The monitoring of the strains throughout the fermentation was carried out by RAPD-PCR. The value of pH was measured by a Foodtrode electrode (Hamilton).

2.5. Determination of carbohydrates and organic acids in sauerkraut

Freeze-dried sauerkraut powder (2 g) was extracted with 20 mL of water/perichloric acid (95:5, v:v). Mixture was sonicated (amplitude 60) using a macro-probe (Vibra-Cell sonicator; Sonic and Materials Inc., Danbury, CT) for 1 min (2 cycles, 30 s / cycle, 5 min interval between cycles) in an ice-bath. The suspension was subjected to stirring conditions at room temperature for 1 h, kept at 4°C overnight, and centrifuged for 10 min at 10,000 rpm. Water-soluble extracts (WSE) were filtered and stored at −20°C until further use. Concentrations of glucose, fructose and mannitol were determined through HPLC analysis equipped with a Spherisorb column (Waters, Millford, USA) and a Perkin Elmer 200a refractive index detector. Elution was at 32 °C, with a flow rate of 1 ml min^{-1}, using acetonitrile 80% as mobile phase. Lactic, acetic and citric acids were determined by HPLC analysis equipped with an Aminex HPX-87H column (ion exclusion, Biorad) and a UV detector operating at 210 nm (Tlais et al., 2021). Elution was at 70 °C, with a flow rate of 0.6 ml min^{-1}, using H_2SO_4 5 mM as mobile phase. Organic acids and sugars standards were purchased from Sigma-Aldrich (Steinheim, Germany).

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2.6. Identification and quantification of free phenolic compounds in sauerkraut

Phenolic compounds analyses were carried out using methanol/water-soluble extracts (MWSE) of samples at the beginning (raw) and the end of fermentation (35, 9 and 7 days for Sp, Seq and T, respectively). Two grams of freeze-dried samples were mixed with 20 ml of methanol/water solution (70:30, v:v) and acidified with hydrochloric acid (0.1%, vol/vol). The use of acidified solvents increases the extraction yield and avoids side reactions. Mixture was sonicated (amplitude 60) using a macro-probe (Vibra-Cell sonicator; Sonic and Materials Inc., Danbury, CT) for 1 min

(2 cycles, 30 s / cycle, 5 min interval between cycles) in an ice-bath. The suspension was incubated at room temperature for 1 h under stirring conditions. The MWSE recovered by centrifugation (10,000 rpm for 10 min) were used after filtration.

LC-MS/MS analysis of 24 free phenolic compounds from MWSE was performed according to the targeted metabolomic method previously designed and validated by Tlais et al. (2021a), by using an UHPLC Dionex 3000 (Thermo Fisher Scientific, Germany) equipped with a Waters Acquity HSS T3 column (1.8 µm, 100 mm × 2.1 mm) (Milford, MA, USA) and coupled to a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Germany) with an electrospray source. Target phenolics were detectable under multiple reaction monitoring (MRM) mode and the compounds were identified based on their reference standard, retention time, qualifier and quantifier ion (Table S2). The management of the chromatographic system and data acquisition was by Xcalibur software version 4.1 (Thermo Fisher Scientific, Germany).

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The use of Waters Acquity HSS T3 column was unsuitable to detect the phenolic acid derivatives. Therefore, separation, determination, and quantification of these derivatives from MWSE were performed according to the method described by Tlais et al. (2021a) through an HPLC system Ultimate 3000 (Dionex, Germering, Germany) as described in subsection 2.2.3.

2.7. Identification and quantification of glucosinolates (GLSs) in sauerkraut

Individual GLSs were extracted from raw cabbage and fermented sauerkrauts as described previously by Palani et al. (2016). Briefly, five millilitres of 70% aqueous methanol were added to 500 mg of freeze-dried powder. The mixture was heated to 70°C with continuous stirring for 5 min. Then the samples were cooled to room temperature and centrifuged for 5 min at 4°C and 4000 rpm. The supernatants were collected, and the extraction process was repeated twice with 2.5 mL

of 70% aqueous methanol. The organic layers were combined, and the solvents were removed by vacuum centrifugation. The dry mass was dissolved in 10 mM ammonium formate (eluent A), filtered with a PTFE filter to make exactly 2 mL final volume and transferred to an HPLC vial and stored at -80°C until analysis.

Separation, determination and quantification of intact glucosinolates were performed by using the method validated by Ediage et al. (2011). Accordingly, the HPLC system Ultimate 3000 (Dionex, Germering, Germany) was used for the chromatographic analysis. A reversed phase Zorbax SB-18 (C18, stable bond), 5 m, 250 mm × 4.6 mm I.D. (Agilent, Diegem, Belgium) column was used for separation of the analytes. The injected volume was 20 µL and column oven was set at 30°C. As mobile phases, water (eluent A) and methanol (eluent B) were used; both containing 10 mM ammonium formate at pH 5.0. A linear gradient program at a flow rate of 1.0 mL min^{-1} was used: 95% A for 1 min, decreasing to 93.1% A between 1 to 10 min; and kept at 93.1% A for 3 min. From 13 to 23 min A was decreased to 79.8%, and finally back to initial conditions (95% A) after 33 min. PDA analyses of intact GLSs were analyzed at 229. Instrument calibration was performed by using the concentration range for all standards from 0.1 to 50 mg L^{-1} with R^2 of more than 0.95. Chromeleon Software version 7 (Dionex, Germering, Germany) was used for instrument control, data acquisition and data analysis.

2.8. Colour analysis of sauerkraut

Color was measured using a Minolta CR-10 Camera. *L*, a*, b** color space analysis method was used, where *L* represents lightness (white-black) and *a* and *b* the chromaticity co-ordinates (red-green and yellow-blue, respectively).

2.9. Texture analysis of sauerkraut

Instrumental firmness of sauerkraut was analyzed on the Texture Analyzer TVT 6700 instrument (Perten instruments, AB) using back-extrusion method, 10 kg load cell. Dimension of the back-extrusion probe is compression plate diameter 45 mm (P-CP45S) coupled with the rig as back extrusion container and holder (R–BECH; RA-C-H50D). Briefly, the back-extrusion container was filled with 75% of sauerkraut sample and placed centrally below the probe. The volume and temperature of the samples was kept constant during the test. The analysis was performed using the TexCalc 5 software provided with the instrument. The test mode settings were set as follows: single cycle compression with sample height 35 mm, distance above sample 45 mm, compression distance 15 mm, pre-test speed 1.0 mm s^{-1}, test speed 1.0 mm s^{-1}, post-test speed 5.0 mm s^{-1}, trigger force 10 g and data acquisition rate of 333 pps (points per second). All the measurements were conducted in triplicates. The texture parameter was calculated using the Force – Distance curve, where maximum positive force is a characteristic of firmness.

2.10. Sensory analysis of sauerkraut

Sensory evaluation of sauerkraut samples was executed using Quantitative Descriptive Analysis (QDA) (Satora, Skotniczny, Strnad, & Piechowicz, 2021) by ten trained panelists. Twelve sensory attributes were evaluated, on a ten-point scale, which were selected in the initial panel discussion. Appearance (color, gloss), odor (raw cabbage, pungent, off-odor), taste (sourness, saltness, sweetness, bitterness, off-taste) and texture (firmness, crunchiness) were considered.

2.11. Statistical analysis

All analyses were performed in triplicates on three biological replicates. Only the phenotypic microarray analyses were performed with two biological replicates for each growth condition. Data were subjected to one-way ANOVA by Statistica for Windows (Statistica7.0 per Windows). Tukey's test was used to determine significant differences among means at an error probability of

5% ($P < 0.05$). Data for acidification, growth, and phenolic compounds metabolism were subjected to the permutation analysis using ClustVis.

3. Results and discussion

3.1. Growth, acidification capability, and metabolism of phenolic compounds of lactic acid bacteria strains in cabbage juice

Twenty-three strains of lactic acid bacteria previously isolated during sauerkraut fermentation were screened based on the growth and acidification capacity and phenolics metabolism in cabbage juice as growth model system. The inoculum cell density was ca. 7.0 log CFU/g and the initial value of pH was 6.06 ± 0.03. After 24 h of incubation, the pH decrease (ΔpH), maximum acidification rate (V_{max}) and lag phase (λ) ranged from 2.26 to 3.04 pH units, 0.15 to 1.03 pH units h^{-1}, and from 0.25 to 5.36 h, respectively (Figure S1). The cell density (A) increased up to 0.42 - 1.43 OD_{620} units. Values of μ_{max} and λ were in the range of 0.04-0.35 OD_{620} units h^{-1} and 0.98-5.53 h, respectively (Figure S1). ~~*Lactobacillus* species~~Lactobacilli showed the highest increase of cell density (1.25-1.43 OD_{620} units) and acidification capacity (2.97-3.04), followed by *Leuconostoc* species (0.91-1.2 and 2.62-2.8, respectively). Whereas *Pediococcus parvulus* strains, excluding *P. parvulus* S2w2, were the less performing strains (Figure S1).

To further screen the pro-technological traits of lactic acid bacteria strains, the profile of free phenolic compounds of fermented cabbage juice was analyzed by HPLC. Phenolic compounds in food have attracted interest because of their health benefits, but also owing to their antimicrobial features, their impact on multiple sensory attributes, and their ability to scavenge free radicals preventing oxidation reactions (Rodríguez et al., 2009; Tlais et al., 2021). It is also increasingly recognized that health benefits of phenolic compounds are partially dependent on their microbial

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conversion through decarboxylase, reductase, glycosyl hydrolase, and esterase activities (Filannino et al., 2018). Under the condition of our study, species- or strain-dependent ability to metabolize phenolic compounds were revealed by using cabbage juice as growth model (Table S3) (Filannino, Gobbetti, De Angelis, & Di Cagno, 2014; Gaur et al., 2020). Caffeic acid, rosmarinic acid, and catechin were the phenolics with the highest abundance (20.59 ± 0.59, 2.3 ± 0.01 and 1.17 ± 0.06 mg L^{-1}, respectively) in unfermented cabbage juice. All strains were able to markedly increase the concentration of caffeic acid (147-660%) and catechin (11-50%), except *P. parvulus* S3w1 for caffeic acid and S5w4 for catechin. Upon fermentation, the levels of other phenolic compounds followed different trends according to the microbial strain. The amount of rosmarinic acid mainly increased with *P. parvulus* S3w8 and S5w4 (50% and 47%) and *L. plantarum* S6w5 (32%), whereas *Leuc. citreum* S7d10 was responsible for the main decrease (45%) compared to unfermented cabbage juice. *L. plantarum* S6w5, *L. paracasei* S4d8, and some *P. parvulus* strains were able to completely metabolize ferulic acid. Solely and almost all *Leuconostoc* strains caused an increase of sinapic acid (350-625%), where the highest concentration was found in cabbage juice inoculated with *Leuc, citreum* S24h3. Only *Pediococcus* strains were able to increase the level of salicylic acid (3-31%). The concentration of isoquercetin doubled when juice was inoculated with *Leuc. mesenteroides* S2d5 and *Leuc. citreum* S2d6, whereas it was completely metabolized by some *Pediococcus* strains. Rutin was found only in cabbage juice fermented with *Leuconostoc* species. The release of dihydroferulic acid was enhanced by most bacterial strains, with *L. plantarum* S6w5 showing the highest increase (105%) (Table S3).

Based on kinetics of acidification and growth, and phenolics metabolism, strains were grouped into 5 clusters (A-E) (Figure 2). *Leuconostoc* species were grouped in clusters A and D, whereas *P. parvulus* strains were grouped in cluster C and E. Cluster B included *L. plantarum* and *L.*

paracasei strains. Assuming that each cluster groups strains with similar behaviour, *Leuc. mesenteroides* S2d5 (cluster A) and S3d1 (cluster D), *P. parvulus* S2w2 (C) and *L. plantarum* S6w5 (B) were selected as representative strains for each cluster. Strains from cluster E were excluded from further characterization due to low kinetic acidification and growth parameters. Utilization of carbon sources was also included as criterion for further screening, thus the carbon consumption profile of selected strains was determined using the Phenotype MicroArray OmniLog PM Technology (Biolog) platform (Figures 3, S2 and S3; Tables S4 and S5). Principal component analysis of the area under the curve (AUC) computed from phenotype profile curves revealed three different clustering patterns of three selected species (Figures 3 and S2; Tables S4 and S5). *Leuc. mesenteriodes* S2d5 and S3d1 showed the same phenotypic profile, distinguished by the high utilization of sucrose, arbutin, amygdalin, D-fructose, α-D-glucose, N-acetyl-D-glucosamine, and palantinose. *L. plantarum* S6w5 consumed mainly glycerol, D-trehalose, maltose, D-methyl-D-glucoside, lactulose, α-D-lactose, and D-sorbitol. On the other hand, L-lyxose, 2-deoxy-D-ribose, D-ribose, D-cellobiose, dihydroxyacetone, gentibiose, maltotriose, and D-gluconic acid were mainly metabolized by *P. parvulus* S2w2 (Figures 3 and S2; Tables S4 and S5). Additionally, the metabolic parameters (AUC, lag phase, maximum absorbance, and metabolic tax) for mainly metabolized carbon sources such as α-D-glucose, α-D-lactose, amygdalin, arbutin, D-fructose, D-manitol, lactulose, salicin, and sucrose were determined (Figure S3). Phenotype profiling revealed the metabolic differentiation among the species and strains, which reflected their potential to follow different metabolic routes and ferment various range of carbohydrates (Gänzle, 2015). Considering the performance and outcome of all the investigated parameters, we selected *Leuc. mesenteroides* S3d1 (high acidification and growth performances, release of catechin and phenolic acids, utilization of certain carbon sources), *P. parvulus* S2w2 (high acidification and growth

performances, release of some phenolic acids and bioconversion of others, utilization of certain carbon sources), and *L. plantarum* S6w5 (highest acidification and growth performances, main producer of phenolic acid derivatives, utilization of many carbon sources) as potential starters for sauerkraut fermentation and used to perform the subsequent fermentation trials.

3.2. Fermentation of sauerkraut

Two sauerkraut starter-assisted fermentation methods were proposed, which included a sequential fermentation (Seq) where the inoculum of three selected lactic acid bacteria strains was temporally deferred, and the ternary fermentation (Ter) through the simultaneous inoculum of all the three strains (Figure 1). For Seq fermentation, the inoculation sequence of bacterial strains and the interval between each inoculation were defined according to the microbial successions and target values of pH, bacteria cell density, and organic acids characterizing spontaneous fermentations previously investigated (Lemos Junior et al., 2022). The persistence of the selected starters throughout the fermentation period was confirmed using the RAPD-PCR profile of bacterial starters during the fermentation period (data not shown). The spontaneous fermentation without microbial inoculum (Sp) and lasting 35 days was used as control. All the methods were carried out at 15°C. Raw cabbage contained 3.97 ± 0.03 Log CFU g^{-1} of presumptive lactic acid bacteria and 3.14 ± 0.12 CFU g^{-1} of yeasts. The initial cell density of all the inoculated strains was ca. 7.0 log CFU g^{-1}. As expected, starter-assisted fermentation ensured an increased number of lactic acid bacteria from the early stages of fermentation (Figure 4). After 1 day, Seq and Ter fermented sauerkrauts had a cell density of lactic acid bacteria respectively of 8.83 ± 0.04 and 9.01 ± 0.02 Log CFU g^{-1}, which remained almost constant throughout the fermentation. Under Sp condition, cell density of presumptive lactic acid bacteria increased ~~their number~~ of ca. 4.5 log cycles during the first 4 days of fermentation ~~after 4 days of fermentation compared to raw cabbages~~, and then

had a downward trend until 21 days, reaching a ~~progressively decreased at~~cell density of ca. 7.0 Log CFU g^{-1} ~~up to 21 days~~. A diauxic growth was observed during the penultimate ~~last two~~ week~~s~~ of spontaneous fermentation (from 21~~8~~ to ~~35~~28 days) reaching a cell density of ca. 8.5 Log CFU g^{-1}. ~~, and t~~The final cell density at the end of fermentation was 7.38 ± 0.12 Log CFU g^{-1} (Figure 4).

The number of yeasts slightly increased after the first days, then decreased progressively to disappear after 21 days of spontaneous fermentation. Seq and Ter fermented sauerkrauts did not show any growth of yeasts in 10 g of samples. Yeasts are considered undesirable microorganisms, especially in the late stages of sauerkraut fermentation and during storage (Yang et al., 2020). They are high producers of pectinase which might be responsible for the softening and deterioration of fermented vegetables (Pino et al. 2019). Yeasts are usually present during the early phase of the sauerkraut fermentation and their growth is highly dependent on the salt concentration in brine, as well as the use of the starter culture, which might explain their absence in Seq and Ter fermented sauerkrauts (Müller et al., 2018).

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3.3. Analysis of pH, carbohydrates and organic acids in sauerkraut

Raw cabbage had an initial pH of 5.48 ± 0.02. A rapid decline in pH (ca. 1.2 units) was observed after one 1 day of fermentation in both starter-assisted fermentations, especially with sequential starter inoculum (Figure 4). The same variation was found after 4 days of spontaneous fermentation. The decrease of pH throughout time reflected the method of fermentation. The pH value of 3.5 was reached in Ter, Seq and Sp fermented sauerkrauts after 5, 9 and 28 days of fermentation, respectively (Figure 4). Glucose (197.2 ± 8.07 mg g^{-1} DM) and fructose (176.3 ± 2.75 mg g^{-1} DM) were the most abundant carbohydrates of raw cabbages. A substantial consumption of glucose was found throughout time in all samples (Figure 4). Small residues of

glucose were found at the end of the starter-assisted fermentations (58.9 ± 5.87 and 78.7 ± 5.62 mg g^{-1} DM in Seq and Ter, respectively), but it is consistent with previous characterizations of traditional sauerkrauts (Yang et al., 2020). The full consumption of fructose occurred after 4 days in Seq fermentation and after 7 days in Sp and Ter fermented sauerkrauts. Fructose was likely used as an alternative external electron acceptor, leading to the release of mannitol throughout time, where the highest levels were found in Ter fermented sauerkrauts (119.4 ± 9.21 mg g^{-1} DM), followed by Seq (97.5 ± 10.66 mg g^{-1} DM) and Sp (80.7 ± 8.24 mg g^{-1} DM) fermented sauerkrauts (Figure 4). Mannitol is known for its antioxidant and sweetener properties (Cui et al., 2019). Synthesis of lactic and acetic acids mirrored the fermentation. The highest level of lactic acid at the of fermentation was found in Ter (80.21 ± 0.01 mg g^{-1} DM), followed by Sp (76.30 ± 0.01 mg g^{-1} DM) and Seq (52.28 ± 0.01 mg g^{-1} DM) fermented sauerkrauts, whereas the synthesis of acetic acid was almost similar among them (Figure 4). Although all fermentation methods had nearly the same maximum acidification capacity, it was at different rates. The Ternary method resulted the most effective in acidifying the sauerkraut in the shortest time. Compared to traditional sauerkraut, shorter acidification process results in a faster transformation of sauerkraut, with less commodity loss and lower production costs (Martorana et al., 2017).

3.4. Identification and quantification of phenolic compounds in sauerkraut

The bioavailability of phenolic compounds was dependent by the fermentation method. Bacterial esterase and glycosyl hydrolase enzymes can release phenolic acids from their conjugated form, which explains the increase of several phenolic acids during sauerkraut fermentations. Aiming to evaluate the effects of different methods of fermentations on free phenolic compounds, their profile was analyzed through LC-ESI-MS/MS and HPLC-PAD analysis. Fifteen phenolic compounds belonging to different chemical classes were identified at the end of fermentation

(Figure 5A). The profile of phenolic compounds and associated derivatives differed significantly ($p < 0.05$) among different fermentations and compared to raw cabbage. Caffeic acid, hydrocaffeic acid and 4 ethyl catechol were not detected in raw cabbage and appeared after the fermentation. Kaempferol and 4-ethyl phenol were the only compounds whose concentration decreased with all fermentation methods. Increases ($p < 0.05$) in the levels of vanillin and *p*-coumaric, sinapic, caffeic, and (E)-cinnamic acids were found in Sp sauerkraut. Focusing on starter-assisted processing, sinapic, 3-hydroxybenzoic, caffeic, ferulic, and (E)-cinnamic acids increased ($p < 0.05$) during Seq fermentation, conversely the same compounds were highly ($p < 0.05$) metabolized when Ter method was used. This reduction with Ter method was associated with the release of phenolic acid derivatives, mainly hydrocaffeic acids, hydroferulic acid and 4-ethyl catechol. Lactic acid bacteria may metabolize phenolic acids, converting caffeic acid to hydrocaffeic acid and 4-ethyl catechol, ferulic acid into hydroferulic acid, sinapic acid into 4-vinylsyringol, and 3-hydroxybenzoic into catechol by using phenolic acid reductases or decarboxylases (Filannino et al., 2014; Nićiforović & Abramovič, 2014; Sengupta, Jonnalagadda, Goonewardena, & Juturu, 2015; Filannino et al., 2020). Compared to their precursors, derivatives exert higher antioxidative and antimutagenic properties, as well act as flavoring agents (Nićiforović & Abramovič, 2014; Septembre-Malaterre, Remize, & Poucheret, 2018). Depending on the phenolic profiles, it seems that the Ter method outperformed the Seq fermentation in terms of phenolic acid derivatives formation. This might be due to the complementarity of enzyme activities of co-cultured bacteria (Canonico, Comitini, Oro, & Ciani, 2016) as well to the prominent role played by *L. plantarum* S6w5, since as the ability to metabolize phenolic acids has been mainly found in lactobacilli (Filannino et al., 2014; Gaur et al., 2020).

3.5. Identification and quantification of glucosinolates (GLSs) in sauerkraut

GLSs, sulfur-rich secondary plant metabolites, are exclusively found in crops belonging to the genus *Brassica* of the family Brassicaceae. They are generated from the amino acid biosynthetic pathway, conferring the pungent and characteristic flavor and odor of *Brassica* plants (Bhandari et al., 2020). Likely phenolic compounds, GLSs and their breakdown products have been shown to exert high biological and pharmacological effects (Martinez-Villalueng et al., 2009; Palani et al., 2016). The effect of fermentation on metabolism of glucosinolates was elucidated by HPLC analysis (Figure 5B.) External standards analyzed under the same conditions were used for the identification by comparing retention time and UV absorbance (Figure 5B and S4). Four aliphatic GLSs (sinigrin, gluconapin, glucobrassicanapin, glucoerucin), one indolyl GLSs (glucobrassicin), two aromatic GLSs (gluconasturtiin, glucotropaeolin), and one GLS derivative (ascorbigen) were identified at concentration that significantly ($p < 0.05$) differentiated raw cabbage from fermented sauerkrauts. Ascorbigen, glucotropaelin, glucobrassicanapin and sinigrin were the most abundant GLS compounds found in raw cabbage (810.4 ± 2.3, 672.8 ± 21.3, 205.3 ± 25.4, 32.9 ± 6.2 µM $100g^{-1}$ DM, respectively). The effect of fermentation varied depending on the method used (Figure 5B). A significant ($p < 0.05$) and high decrease of glucobrassicanapin, glucobrassicin and ascorbigen was found in Sp and Seq fermented samples, whereas increased amounts for the same compounds were found under Ter fermentation condition. An opposite trend was found for gluconasturtiin. The levels of sinigrin and glucotropaelin mainly decreased after Sp fermentation. The sinigrin-metabolizing activity in lactic acid bacteria was previously described by Watanabe et al. (2021) and is induced under glucose-absence, which can explain the lower level of sinigrin under Sp fermentation compared to Ter and Seq (Figure 5B). Compared to raw cabbages, fermentation led also to higher amounts of glucoerucin and gluconapin, especially under Sp fermentation (Figure 5B).

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According to the literature, most studies agreed that spontaneous or starter-assisted sauerkraut fermentation has a significant effect on the hydrolysis of GLSs and release of their derivatives (Martinez-Villalueng et al., 2009; Mullaney, Kelly, McGhie, Ansell, & Heyes, 2013; Palani et al., 2016; Narbad & Rossiter, 2018), but the direction of GLSs hydrolysis or conversion depends on the fermentation conditions (Sikorska-Zimny & Beneduce 2020, Sikorska-Zimny & Beneduce 2021). According to several studies, the major end-product of indole GLSs in sauerkraut is ascorbigen, which is one of the most powerful bioactive compounds in *Brassica* plants (Martinez-Villalueng et al., 2009; Palani et al., 2016). In our study, fermented sauerkrauts with longer incubation periods (Sp and Seq), showed simultaneous degradation of glucobrassicin and ascorbigen. The chemical instability of ascorbigen toward acids, bases, and temperatures could explain its low concentrations in these longer fermented sauerkrauts (Opietnik et al., 2012). On the other hand, the higher levels of glucobrassicin, ascorbigen, and glucobrassicanapin under Ter fermentation might reflect the high metabolic potential of the Ter method, for instance in releasing bound GLSs. The duration of fermentation and the metabolic activities of the microorganisms involved likely also guided the increase of glucoerucin level during sauerkraut fermentation, especially under Sp condition. We have to point out that some lactic acid bacteria are also responsible for the interconversion of certain GLSs, such as the reduction of glucoraphanin into glucoerucin (Mullaney et al., 2013). Our results clearly indicated that the timing of inoculation had a significant effect on the profiles of GLS, as well as phenolics, and can be used to manipulate the profile of bioactive compounds in sauerkraut and to develop ad-hoc fermented products for health promoting purpose.

3.6. Evaluation of color, texture and sensory analysis in sauerkraut

Color indices and texture of raw cabbage and sauerkrauts at the end of fermentation were shown in Table 1. *L** values indicate colour lightness, and high values are desirable for sauerkraut~~Color lightness~~. In our study, ~~(~~*L**~~)~~ did not significantly ($p > 0.05$) differ among samples. As expected, the scaler quantity of red-green (*a**) showed negative values for all samples, especially in raw cabbage. The yellow-blue (*b**) index of Sp fermented sample was significantly ($p < 0.05$) higher than those found in the raw cabbage, while slightly decreased when the Ter fermentation was adopted. The firmness (N), one the most essential texture attributes of the sauerkraut, was measured by Texture Analyzer TVT 6700 instrument (Table 1). A considerably ($p<0.05$) minimal force (N) was measured in Sp fermented sauerkrauts suggesting a decrease of firmness and crunchiness compared to raw cabbage. On the contrary, the firmness of Seq and Ter fermented sauerkrauts was similar to the raw cabbage, indicating a preservation effect mediated by starter-assisted fermentation, which may be attributable to complete inhibition of the yeasts growth. Softness and low crunchiness of the sauerkraut is usually poor desirable for sauerkraut.

At the end of fermentation, all samples were subjected to sensory analysis (Table 1). Twelve sensory attributes concerning appearance, odor, taste, and texture of all fermented sauerkrauts were evaluated on a ten-point scale. The score of most of the sensory attributes did not significantly ($p > 0.05$) differ among samples. Focusing on the distinctive features, glossiness distinguished Sp and Seq (5.5 ± 1.4 vs 4.1 ± 1.0), whereas Sp and Ter were differentiated for sourness (5.9 ± 1.2 vs 4.2 ± 1.6) and sweetness (2.2 ± 1.1 vs 3.6 ± 1.4) scores. Raw cabbage note was evaluated at higher ($P < 0.05$) level (5.1 ± 0.7) in Ter sauerkraut compared to Sp (2.2 ± 0.6) and Seq sauerkraut (2.8 ± 0.6). The distinctive sensorial features of sauerkraut fermented with the three methods were the consequence of the different profiles of sugars, organic acids, phenolic compounds, and GLSs, which largely determines the organoleptic properties of sauerkraut (Satora et al., 2021). For

instance, the perception of a sour taste can be affected by the presence of sugars and polyols, in addition to organic acids. GLSs and their derivatives originating during fermentation are considered among the main responsible of the characteristic flavor of sauerkraut. It was expected that short fermentation periods, such as that of Seq and Ter, would alter the typical organoleptic profile of long spontaneously fermented sauerkraut. Surprisingly, our results showed only slight changes in the sensory profile, no significant impact on colour lightness, and a preservative effect on the structural integrity of sauerkraut.

4. Conclusion

Under our investigation, a varied pool of lactic acid bacteria involved in sauerkraut fermentation was screened based on a diversified panel of metabolic parameters, revealing species- or strain-dependent metabolic traits inherent in the ability to metabolize phenolic compounds and to ferment various range of carbohydrates in cabbage juice model. Three high performing starter candidates were selected for sauerkraut processing and used to develop effective steering fermentation methods. Started assisted fermentations of sauerkraut overcame the drawback associated to a long fermentation, especially using simultaneously ternary cultures. Furthermore, we demonstrated how combined ternary cultures enhanced phenolic compound conversion by complementing enzyme activities, ensuring at the same time higher level of aliphatic (glucobrassicanapin) and indole GLSs (glucobrassicin) and GLSs derivatives (ascorbigen). Thus, selected ternary cultures can be used to manipulate the profile of bioactive compounds in sauerkraut in order to develop ad-hoc fermented products for health promoting purpose, limiting at the same time the negative consequences of a short fermentation on the typical organoleptic profile of sauerkraut.

CRediT authorship contribution statement

Ali Z.A. Tlais: Methodology, Investigation, Formal analysis, Writing - original draft. **Sadia Kanwal:** Methodology, Investigation, Formal analysis, Writing - original draft. **Pasquale Filannino:** Conceptualization, Methodology, Writing - review & editing. **Marta Acin Albiac:** Investigation, Formal analysis. **Marco Gobbetti:** Supervision, Writing - review & editing. **Raffaella Di Cagno:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Writing - original draft, Writing - review & editing.

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Conflict of 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.

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Figure 1. Schematic representation of the sauerkraut fermentation methods adopted in this study.

Figure 2. Pseudo-heat map showing the acidification (ΔpH, V_{max}, λ_o), growth (A, μmax, λ) parameters, and the variation of the concentration of phenolic compounds in cabbage juice inoculated with lactic acid bacteria strains for 24 h at 30°C. The variation of the concentration was compared to that of raw cabbage juice. Rows are clustered using Euclidean distance and McQuitty linkage. The color scale shows differences, with brown and blue indicating the highest and lowest values of the standardized data.

Figure 3. Principal component analysis (PCA) of the area under the curve (AUC) computed from phenotype microarray data as determined by Biolog system. *Lactiplantibacillus plantarum* S6w5, *Pediococcus parvulus* S2w2, or *Leuconostoc mesenteriodes* S2d5 and S3d1 were inoculated in cabbage juice incubated at 30°C for 24 h. Loadings represent the consumption of C sources across the PCA space.

Figure 4. Microbiological, physical and biochemical characterization of sauerkrauts during the spontaneous (Sp), starter-assisted sequential (Seq) and ternary (Ter) fermentation at 15°C. *Leuconostoc mesenteriodes* S3d1, *Pediococcus parvulus* S2w2 and *Lactiplantibacillus plantarum* S6w5 were the selected starters, which were inoculated according to the fermentation methods described in the Materials and Methods. The data are the means of three independent experiment ± standard deviations (n=3).

Figure 5. Quantification of phenolic compounds (μg g^{-1} DM) by LC-ESI-MS/MS and HPLC-PAD (A) and glucosinolates (μmol 100 g^{-1} DM) by HPLC-PAD (B) in extracts obtained from raw cabbage (Raw), spontaneous (Sp), starter-assisted sequential (Seq), and ternary (Ter) sauerkrauts fermentation at 15°C. *Leuconostoc mesenteriodes* S3d1, *Pediococcus parvulus* S2w2 and

Lactiplantibacillus plantarum S6w5 were the selected starters, which were inoculated according to the fermentation methods described in the Materials and Methods.

Data were subjected to one-way ANOVA followed by Tukey's procedure at $p < 0.05$. Bars with different superscript letters differ significantly ($p < 0.05$).

Supplementary data

Figure S1. Parameters of growth and acidification kinetics of 23 strains of lactic acid bacteria during fermentation of cabbage juice at 30°C for 24 h.

Growth and acidification kinetics were modelled according to the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, & Van't Riet (1990): $y = k + A$ or $\Delta pH \exp\{-\exp[(\mu_{max}$ or V_{max} e/A or $\Delta pH)(\lambda - t) + 1]\}$. When the optical density (OD_{620}) was the dependent variable to be modelled, k was the initial level of optical density (OD_{620} units), A was the difference in OD_{620} units between inoculation and the stationary phase, μ_{max} was the maximum growth rate (OD_{620} units h^{-1}), λ was the length of the lag phase (hours), and t was the time (hours). When the pH (pH units) was the dependent variable to be modelled, k was the initial level of pH, ΔpH was the difference in pH units between inoculation and the stationary phase, V_{max} was the maximum acidification rate (pH h^{-1}), λ was the length of the lag phase (hours), and t was the time (hours).

Figure S2. Heatmap showing the area under the curve (AUC) computed from phenotype microarray data as determined by Biolog system. *Lactiplantibacillus plantarum* S6w5, *Pediococcus parvulus* S2w2, or *Leuconostoc mesenteriodes* S2d5 and S3d1 were inoculated in cabbage juice incubated at 30°C for 24 h. The color scale shows differences in term of AUC, with a gradient from yellow to blue indicating the lowest and highest values of the AUC, respectively.

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Each phenotype profile was assayed for growth in the presence of various carbon using Omnilog *Phenotype MicroArray*, as described in the Materials and Methods.

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Figure S3. Metabolic parameters for carbon sources mainly metabolized by *Lactiplantibacillus plantarum* S6w5, *Pediococcus parvulus* S2w2, and *Leuconostoc mesenteriodes* S2d5 and S3d1 inoculated in cabbage juice incubated at 30°C for 24 h. (A) Area under the curve (AUC), (B) lag phase (h), (C) maximum absorbance, and (D) metabolic tax (h^{-1}).

Figure S4. Separation by HPLC (229 nm) of glucosinolates in extracts from raw cabbage (Raw), spontaneous (Sp), starter-assisted sequential (Seq), and ternary (Ter) sauerkrauts fermentation at 15°C. *Leuconostoc mesenteriodes* S3d1, *Pediococcus parvulus* S2w2 and *Lactiplantibacillus plantarum* S6w5 were the selected starters, which were inoculated according to the fermentation methods described in the Materials and Methods.

Peak assignments: 1, Sinigrin hydrate; 2, Gluconapin; 3, Glucobrassicanapin; 4, Glucotropaelin; 5, Glucoerucin; 6, Glucobrassicin; 7, Gluconasturtiin; 8, Ascorbigen.

Table 1. Color indices (*L**, *a**, and *b**, white-black, red-green and yellow-blue coordinates, respectively), texture (N), and sensory analysis of raw cabbage (Raw) and sauerkrauts during the spontaneous (Sp), starter-assisted sequential (Seq) and ternary (Ter) fermentation at 15°C. *Leuconostoc mesenteriodes* S3d1, *Pediococcus parvulus* S2w2 and *Lactiplantibacillus plantarum* S6w5 were the selected starters, which were inoculated according to the fermentation methods described in the Materials and Methods. Data were subjected to one-way ANOVA followed by Tukey's procedure at $p < 0.05$.

	Color indices			**Texture**	**Sensory properties**											
	*L**	*a**	*b**	Firmness (N)	Colour	Gloss	Raw cabbage	Pungent	Off-odor	Sourness	Saltness	Sweetness	Bitterness	Off-taste	Firmness	Crunchiness
Raw	51.25 ± 1.43^{a}	-8.1 ± 0.21^{a}	19.37 ± 1.31^{b}	691 ± 24.04^{a}	-	-	-	-	-	-	-	-	-	-	-	-
Sp	51.91 ± 1.34^{a}	-6.03 ± 0.21^{b}	24.03 ± 1.62^{a}	490 ± 79.19^{b}	6.2 ± 1.3^{a}	5.5 ± 1.4^{a}	2.2 ± 0.6^{b}	4.5 ± 1.5^{a}	2.2 ± 1.6^{a}	5.9 ± 1.2^{a}	5.2 ± 1.5^{a}	2.2 ± 1.1^{b}	2.2 ± 0.9^{a}	0.9 ± 0.9^{a}	5.3 ± 0.7^{a}	5.6 ± 1.3
Seq	52.34 ± 2.01^{a}	-6.26 ± 0.47^{b}	18.13 ± 1.39^{b}	642 ± 16.97^{ab}	5.1 ± 1.3^{a}	4.1 ± 1.0^{b}	2.8 ± 0.6^{b}	3.5 ± 1.8^{a}	2.6 ± 1.6^{a}	5.4 ± 1.8^{ab}	4.1 ± 1.5^{a}	3.2 ± 1.5^{ab}	1.4 ± 1.0^{a}	1.1 ± 0.9^{a}	5.7 ± 1.3^{a}	6.1 ± 1.3
Ter	53.50 ± 1.29^{a}	-6.05 ± 0.22^{b}	14.38 ± 0.85^{c}	652 ± 28.28^{ab}	5.7 ± 1.2^{a}	5.2 ± 1.2^{ab}	5.1 ± 0.7^{a}	3.7 ± 1.7^{a}	3.6 ± 1.3^{a}	4.2 ± 1.6^{b}	3.8 ± 1.5^{a}	3.6 ± 1.4^{a}	1.2 ± 1.1^{a}	1.2 ± 0.6^{a}	5.9 ± 1.1^{a}	5.9 ± 1.0

(-): not analyzed

$^{a-b}$ Means within the columns with different superscript letters are significantly different ($p < 0.05$). Means within the columns with the same superscript letters are not significantly different ($p > 0.05$).

The data are the means of three independent experiment ± standard deviations (n=3).

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Figure 1

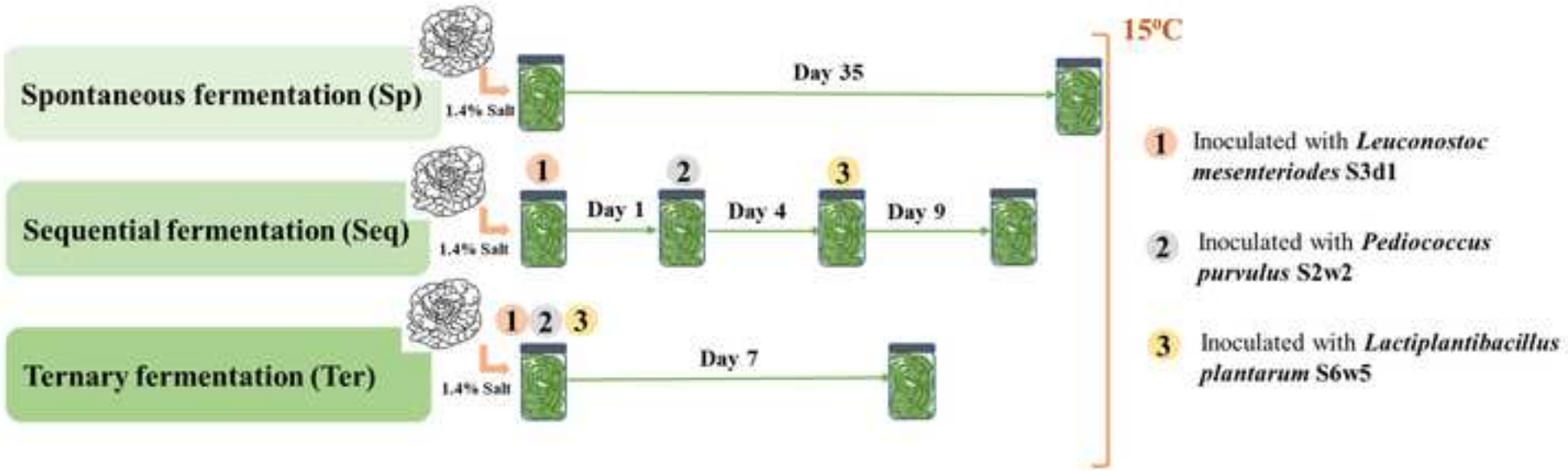

Figure 2

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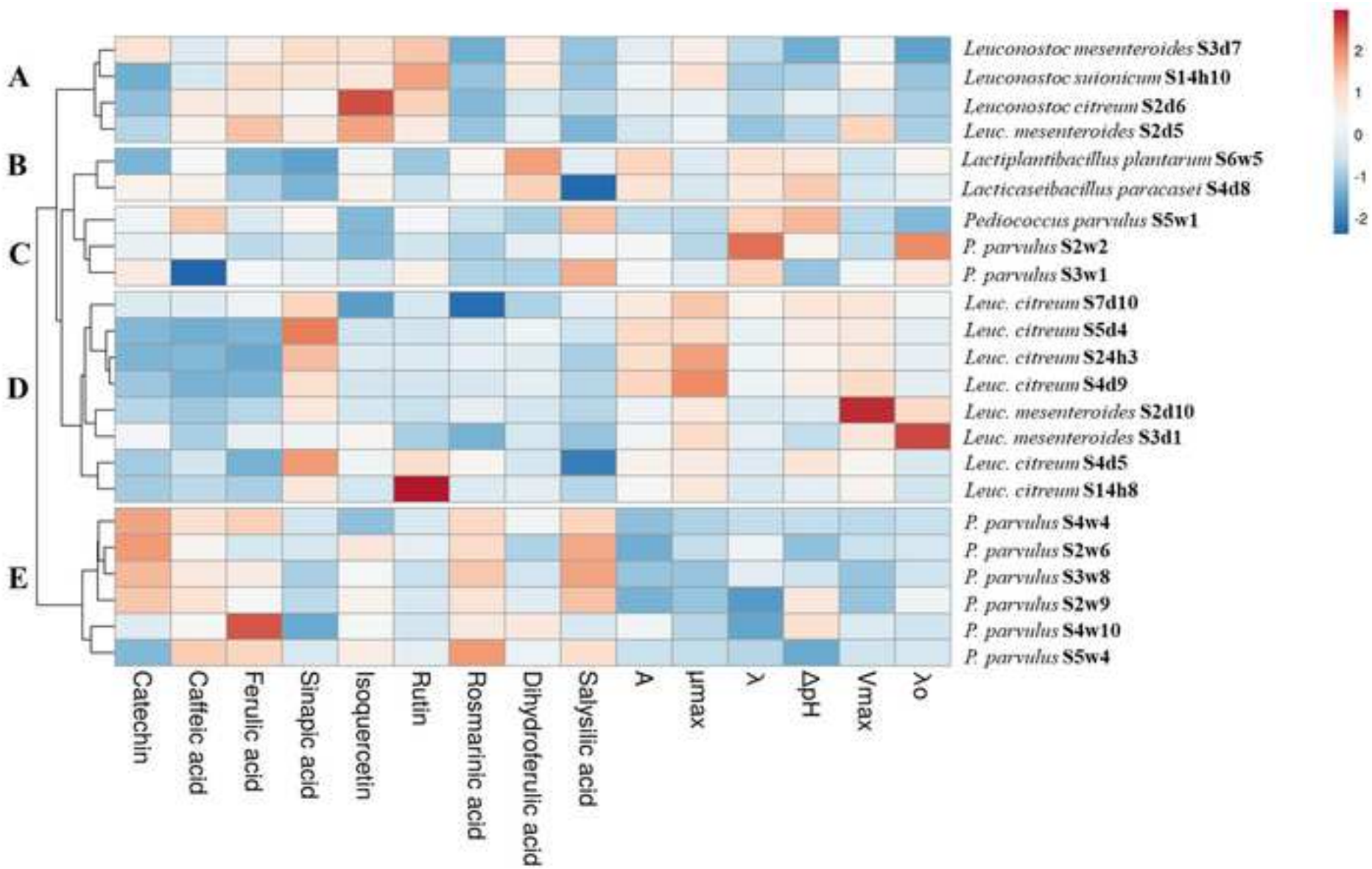

Figure 3

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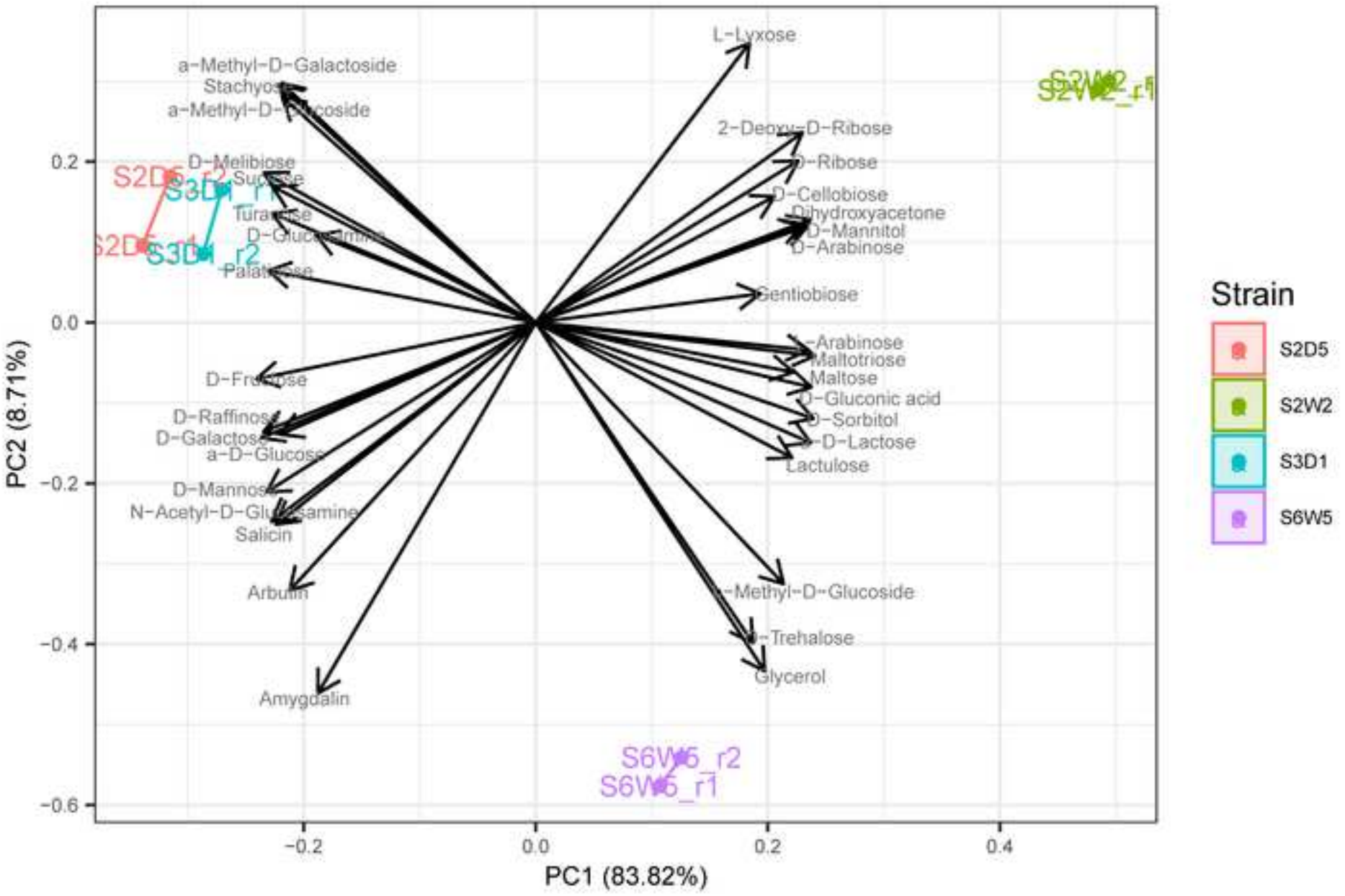

Figure 4

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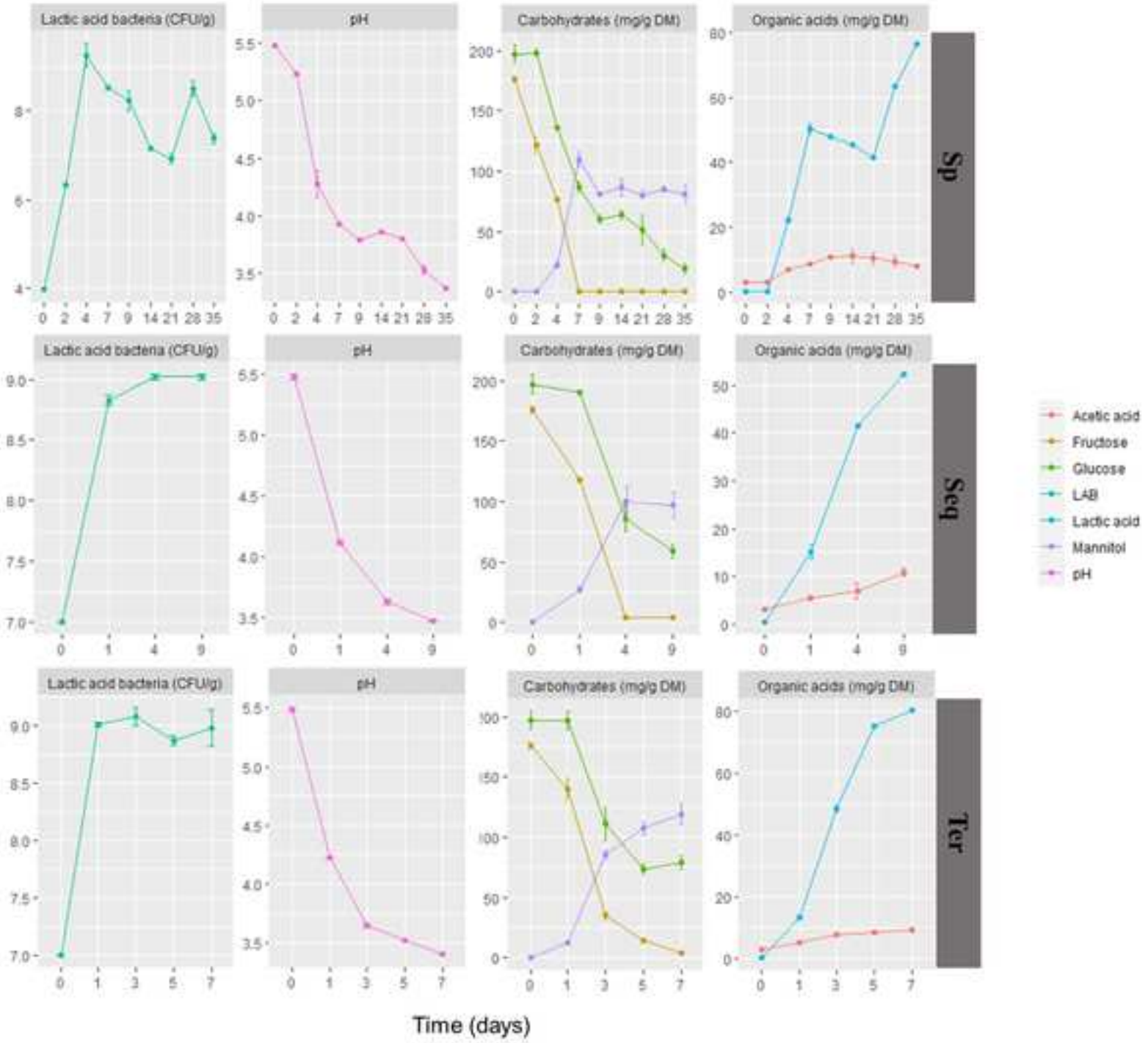

Figure 5A

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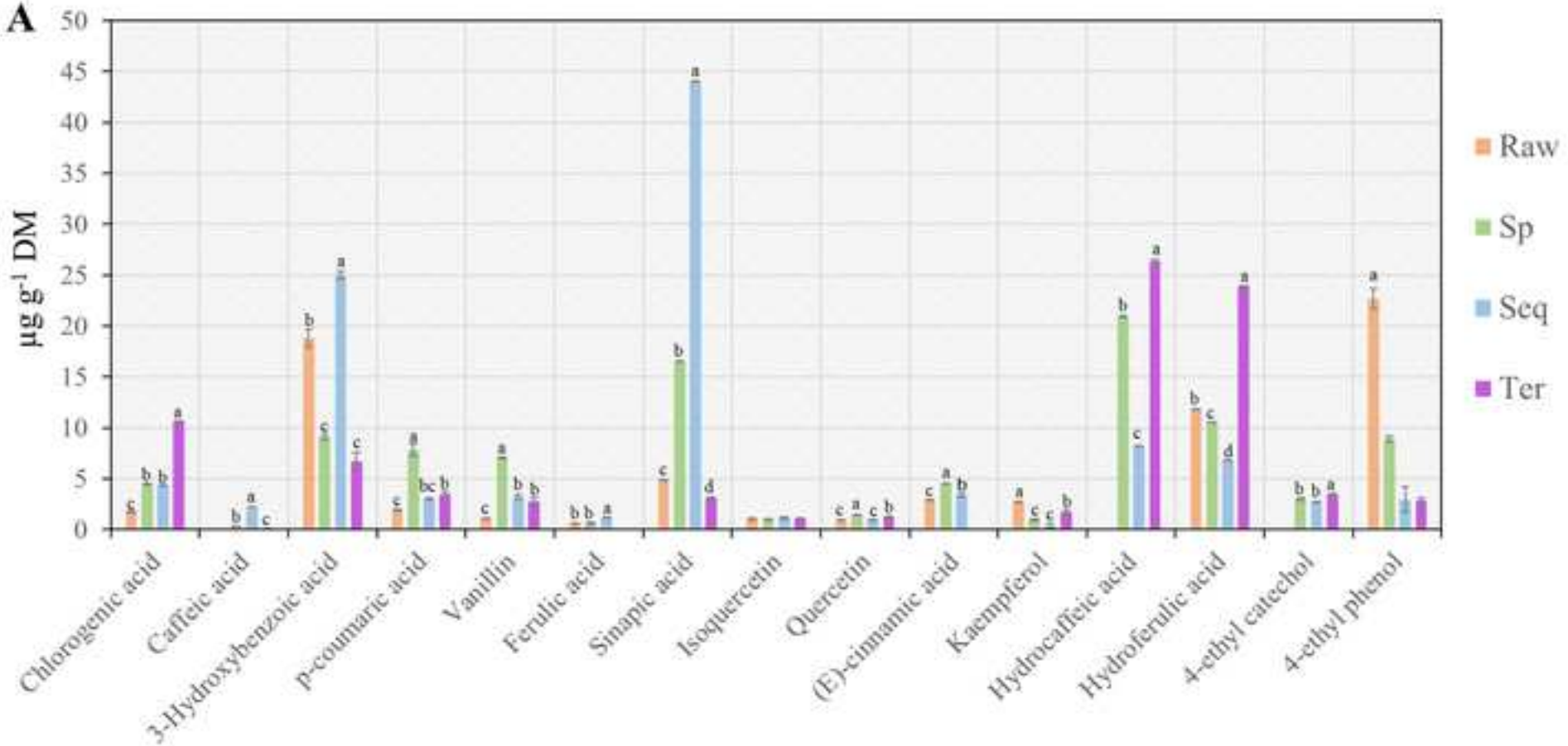
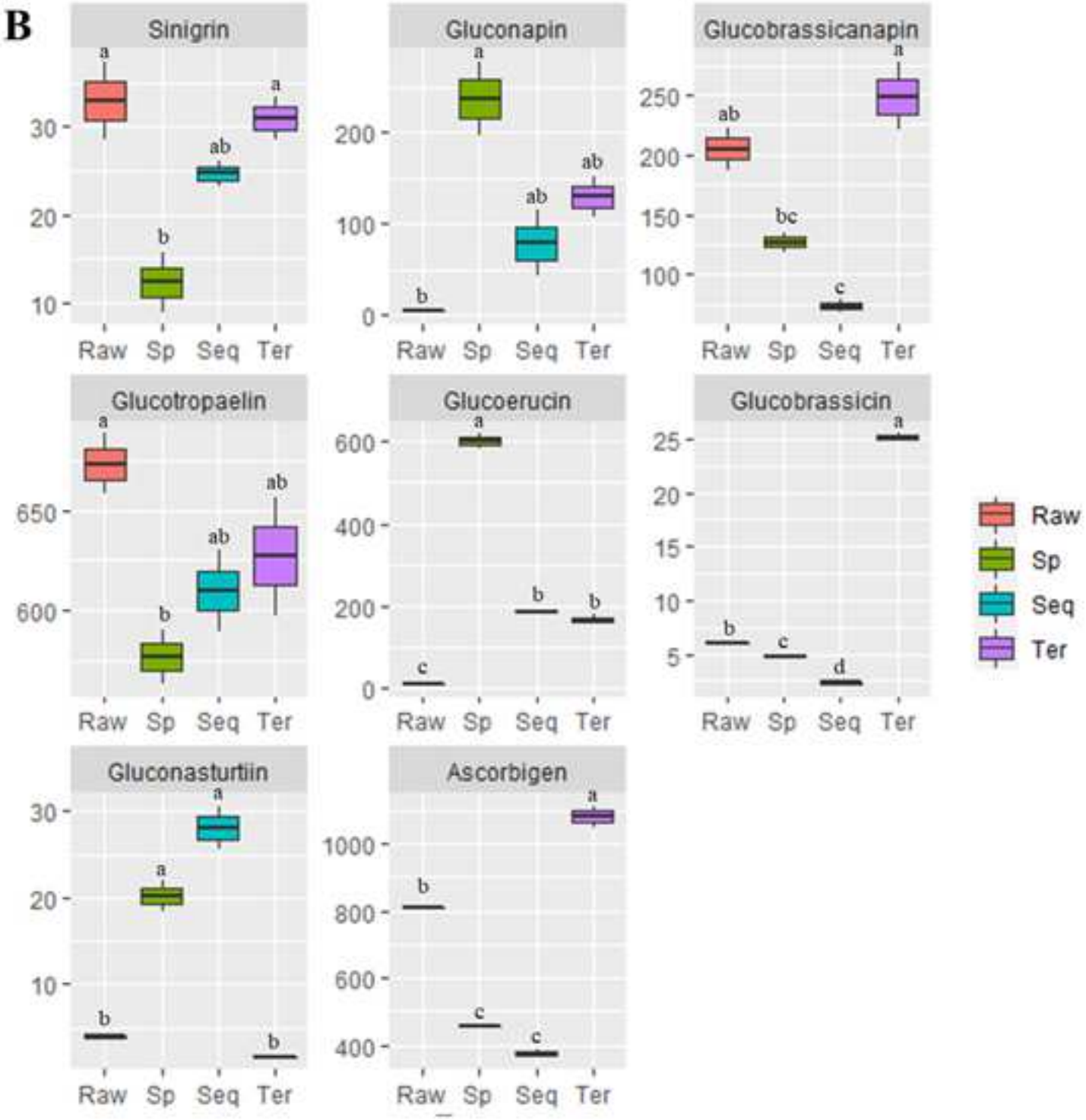


Figure 5B



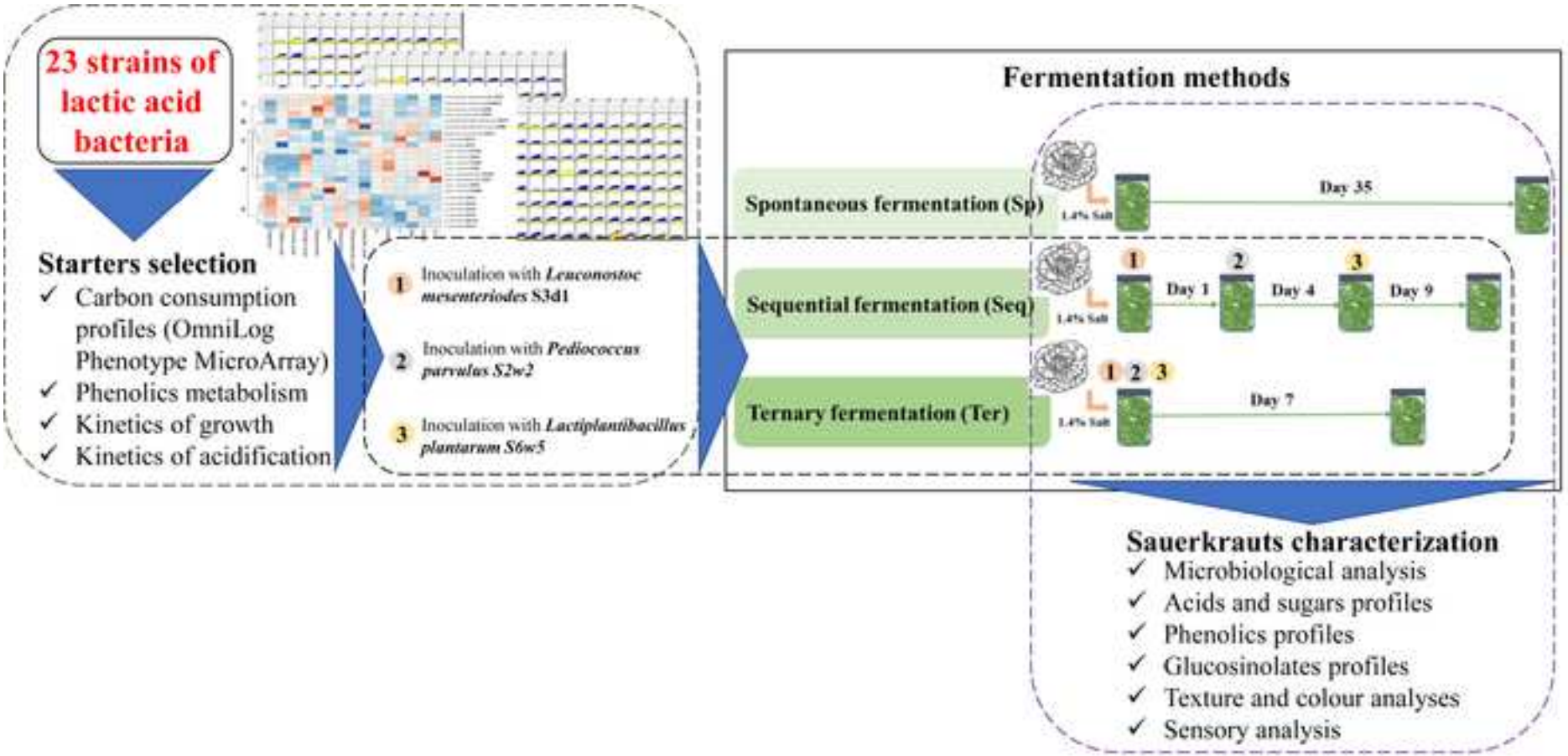
Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

Supplementary Figure S4

Graphical Abstract



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Ali Z.A. Tlais: Methodology, Investigation, Formal analysis, Writing - original draft. **Sadia Kanwal:** Methodology, Investigation, Formal analysis, Writing - original draft. **Pasquale Filannino:** Conceptualization, Methodology, Writing - review & editing. **Marta Acin Albiac:** Investigation, Formal analysis. **Marco Gobbetti:** Supervision, Writing - review & editing. **Raffaella Di Cagno:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Writing - original draft, Writing - review & editing.

Declaration of interests

x 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: