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Prebiotic potential and bioactive volatiles of hemp byproduct fermented by lactobacilli.

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Abstract:	Plant-based feedstock nutritionally and functionally rich are evermore requested in the food industry, although sustainability is a must. An untapped and sustainable source is hemp seed bran (HPB), which is a byproduct of industrial hemp seed flour. In this research we have studied the fermentation of HPB with different beneficial bacteria with the intention to valorize HPB for further food applications as a fiber supplement. Prebiotic activity was tested in vitro, and microbiological features were monitored and studied, as fermentation process and release of volatile organic compounds (VOCs). Results indicate that fermentation is able to increase terpenes and organic acids of HPB, particularly when is conducted by a bacterial pool. Besides, p -Cymene, Myrcene, and Eugenol are the VOCs majorly correlated to prebiotic activity. Although other studies must be conducted, this paper suggests that HPB should be valorized as a substrate to produce sustainable and chemical free prebiotics.

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Prebiotic potential and bioactive volatiles of hemp byproduct fermented by lactobacilli.

Highlights

- **Hemp seed bran is an untapped food source for human consumption**
- **Prebiotic activity was studied analyzing microbial growth and bioactives production**
- **A bacterial pool fermented better hempseed bran than single bacterial species**
- **Volatile SCFAs and terpenes of bran are increased with lactobacilli fermentation**
- **Fermentation improves prebiotic potential of hemp seed bran**

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Prebiotic potential and bioactive volatiles of hemp byproduct fermented by lactobacilli.

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Highlights

- **Hemp seed bran is an ~~unexploited~~ untapped food source for human consumption**
- **P~~The p~~rebiotic ~~potential~~activity ~~is studied coupling~~was studied analyzing microbial growth ~~microbiology~~ and bioactives production**
- **A bacterial pool fermented better hempseed bran than single bacterial species~~The best fermentations of hemp seed bran are those made with a bacterial pool.~~**

- **Volatile SCFAs and terpenes of bran are increased with lactobacilli fermentation**
- **Fermentation improves prebiotic potential of hemp seed bran**

Abstract

Plant-based feedstock nutritionally and functionally rich are evermore requested in the food industry, although sustainability is a must. An untapped and sustainable source is hemp seed bran (HPB), which is a byproduct of industrial hemp seed flour. ~~This research concerns over the exploration of prebiotic activity of hemp seed bran and its exploitation throughout fermentation by beneficial lactobacilli~~In this research we have studied the fermentation of HPB with different beneficial bacteria with the intention to valorize ~~Its aim is to shed light on hemp seed bran~~HPB for further food applications as a fiber supplement ~~in foods~~. Prebiotic activity was tested *in vitro*, and microbiological features were monitored and studied, as fermentation process and release of volatile organic compounds (VOCs). ~~The assessment of its prebiotic activity, investing over bacterial growth and prebiotic related volatilome, r~~Results~~ed~~ indicate that fermentation is able to ~~higher scores with fermentation by increment in~~increase terpenes and organic acids of HPB, particularly when is conducted by a bacterial pool. Besides, *p*-Cymene, Myrcene, and Eugenol ~~were~~ are ~~those~~ the VOCs~~compounds~~ majorly correlated to prebiotic activity. ~~Though the hemp seed value is well known,~~ Although other studies must be conducted, ~~wealthy byproducts hitherto scarcely studied should be valorized, and this~~this paper suggests that HPB should be valorized as a substrate ~~work vows to provide some basics~~ to produce sustainable and chemical free prebiotics.

Keywords

Cannabis sativa sativa; bran; metabolomics; multivariate analysis; *p*-Cymene

Myrcene (PubChem CID:31253); p-Cymene (PubChem CID:7463); Acetic Acid (PubChem CID:176); Propionic Acid (PubChem CID:1032); Butyric Acid (PubChem CID:264); 1-Octen-3-ol (PubChem CID:18827); Eugenol (PubChem CID:3314); Terpinolene (PubChem CID:11463); Myrtenal (PubChem CID:61130); Fenchyl Alcohol (PubChem CID:6997371).

1. Introduction

Hemp (*Cannabis sativa* subsp. *sativa*) is the ~~non-drug~~ variety with no ~~and contains legal content of~~ psychotropic ~~agent~~ effect (Korus, Witczak, Ziobro & Juszczak, 2017). The food products derived are steering a large sector market that is constantly rising worldwide up to~~. The global sector expected to generate around~~ USD 4.89 billion by 2026 ~~(at a CAGR (Compound Annual Growth Rate) of around 6.2% between 2019 and 2026 (~~Zion Market Research 2018). This high rise is due to the ease on ~~legal~~ restraints for registered varieties with no psychotropic effect, considering plants cultivation and transformation and consumption of derived products. The feedstock principally exploited in the hemp food industry are the seeds, that are free of cholesterol, are rich in proteins, vitamins, and minerals, are plenty of dietary fibers and bioactives (Hartsel, Eades, Hickory & Makriyannis, 2016; Wang, Jiang & Xiong, 2018; El Sohly, Radwan, Gul, Chandra & Galal, 2017) and do not contain any psychotropic agent. Hemp seeds are rich in ~~T~~terpenes ~~of hemp have~~with ~~outstanding~~ antioxidant activity (Frassinetti et al., 2018) and their use is regulated as ~~are~~ flavor and fragrance components generally regarded as safe (GRAS) ~~by several regulatory agencies~~ (Hao, Gu & Xiao, 2015). Hemp seeds are transformed in flour ~~is~~ that is ~~principally produced~~used for human consumption, while the proteinaceous cake is used for animal feeds, and t~~. Whatever the industrial process, t~~he derived bran is a byproduct mainly discarded, but possibly ~~represents a high value material to suit~~still valid for further food applications. A specific address could be that of prebiotics. The current definition is stating that “a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017), and this version enlarged the concept to other compounds than traditional polysaccharides. Consequently, complex substrates as

dietary fibers, that bring and liberate or serve for the gut microbiota to generate different bioactive molecules ~~such as~~ (short chain organic acids or terpenes), could attain to this new description. The para prebiotic activity of some fibers could be improved with *in vitro* fermentation by beneficial bacteria. This strategy permits to obtain a product with~~Besides, it is important to consider that fermentation by lactobacilli improves the quality of the final product substantially over two aspects: firstly, the deconstruction of the fiber liberates other compounds, such the so-called postbiotics, that improves the prebiotic potential of the original product, while secondly the yield and~~more bioactives and to improve their bio accessibility ~~of many different bioactives~~ (polyohenols and terpenes) ~~resulted augmented~~. These bacteria are able to ferment plant-based matrices generating and transforming metabolites. ~~For~~ For instance, ~~when~~ *Lacticaseibacillus rhamnosus*~~*Lactobacillus rhamnosus*~~ LGG ~~is applied in combination~~and different ~~with two~~ *Lactiplantibacillus plantarum subsp. plantarum* ~~*Lb. plantarum*~~ isolates ~~to ferment plant-based products,~~ improve~~s~~ the content of phenols and flavonoids of blueberry pomace (Yan et al., 2019). ~~In fact, the enzymatic arsenal of lactobacilli, such as that of~~Strains of *Lp. plantarum*~~*Lb. plantarum*~~, *Lc. rhamnosus*~~*Lb. rhamnosus*~~, and *Limosilactobacillus fermentum* improve the content of bioactives of hemp seed products ~~*Lb. fermentum* perfectly conveys them for fermentation of plant-based matrices~~ (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; Nissen, di Carlo & Gianotti, 2020). Additionally, these species are beneficial, and some related strains own the claim of probiotics, as ~~*Lb. rhamnosus GG* (~~LGG~~)~~, *Lp. plantarum*~~*Lb. plantarum*~~ K10, and *Lm. fermentum* ~~*Lb. fermentum*~~ ME-3 (Darby, Naudin, Luo & Jones, 2019: Kim, Huang, Park, Holzapfel & Lim, 2018; Nowak, Paliwoda & Błasiak, 2019).

Up today, no works were conducted exploring the functional properties of hemp seed bran (HPB) after fermentation. Due to this reason, we made ~~T~~this work with the intention to ~~explored~~ characterize and valorize ~~d hemp seed bran~~HPB ~~(HPB)~~ potential functionalities as a consequence of LAB (lactic acid bacteria) fermentation. ~~by coupling its~~We aimed to achieve this goal by coupling prebiotic activity ~~to~~ to the release of potential ~~volatile organic~~ bioactives volatile organic compounds (VOCs)~~as a consequence of LAB fermentation: i)~~, such as low organic acids, ~~coming~~

directly from microbial metabolismpostbiotics produced by LAB fermentation, and ii) terpenes, more related tointrinsic compounds of hemp seed bran matrixcompounds modification. We have compared HPB to a positive control, i.e. fructooligosaccharides (FOS) from chicory, as the most renowned prebiotics. The This work wants to give more details on the valorization of HPB for food applications that resulted still unexplored novelty of this work has to be found in the byproduct tested, that up to now results poorly characterized (Setti et al.Samaei, Maggiore, Nissen, Gianotti & Babini, 2020) employing emerging approaches to study, improve, and highlight and on the methodology used to assay prebiotic activity,the prebiotic potential of HPB based on a combination of molecular methods to quantify bacterial cells and bioactive VOCs.

2. Materials and methods

2.1. HPB Hemp seed bran preparation

HPB, a byproduct remaining after mechanical pressing of hemp seeds and subsequent grinding and sieving, was supplied by a local company (Hemp Positive World, Cesena, Italy). Original hemp variety was Futura 75. Five grams of HPB were suspended in 30 mL of distilled water, sterilized (121 °C and 100 kPa for 20 min) (Vapor Matic 770, ASAL Srl, Milan, Italy) in independent 50 mL Falcon conical tubes (Corning Inc., NY, USA)50 mL plastic tubes (121 °C and 100 kPa for 20 min), named TBH (Treated Bran Hemp), and then used as substrate for bacterial fermentations, named FBH (Fermented Bran Hemp). Before fermentation addition of the bacterial inoculum, the suspension was adjusted to 27 mL of volume, in order to add later just 3 mL of bacterial inoculum. Controls used were: i) not inoculated sterile HPB/water suspension (NF = Not Fermented); ii) not sterilized nor inoculated sample (BH = Bran Hemp); iii) and a commercial hemp seed flour (HF) (Hanf & Natur, Lindlar, Germany) in a similar water suspension.

2.2. Microbial strains and culture conditions

All microbial strains tested belong to the microbial collection of DISTAL (Dept. of Agricultural and Food Sciences), University of Bologna (Bologna, Italy) and have been previously isolated from plant-based products and extensively studied (~~Babini, Tagliazucchi, Martini, Dei Più & Gianotti, 2017;~~ Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; Babini, et al. 2020; Nissen, di Carlo & Gianotti, 2020~~; Nissen, Casciano & Gianotti, 2021~~). *Lactiplantibacillus plantarum subsp. plantarum* 98b, *Limosilactobacillus fermentum* MR13, *Lacticaseibacillus rhamnosus* C1112 (used for hemp bran fermentation), *Bifidobacterium bifidum* NCIMB 700795 and *Escherichia coli* ATCC 25922 (used for prebiotic activity) were cultured from glycerol stocks stored at -80 °C and were propagated in selective media (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at specific conditions (Nissen, di Carlo & Gianotti, 2020).

2.3. Fermentations

The hemp seed bran samples were fermented independently by *Lc. rhamnosus* C1112 (C), *Lp. plantarum* 98b (L), *Lm. fermentum* MR13 (M), and by a bacterial pool (P) containing equal proportion of the aforementioned strains. Cell load of inoculated bacteria was standardized by spectrophotometric means based on plate counts and qPCR (quantitative PCR). I~~. For each inoculum~~a were made by ~~three~~ 3 mL of 7 Log_{10} cells/mL of bacterial cells, ~~were~~ centrifugated and resuspend two times in sterile distilled water. Each inoculum was added to 27 mL of TBH ~~be~~suspension ~~fore addition to TBH, whose fermentation was conducted in 30 mL. Fermentation~~ and fermentation was conducted aerobically at 37 °C up to 72 h in 50 mL Falcon conical tubes (Corning, USA) ~~aerobically at 37 °C up to 72 h~~ to obtain FBH ~~(Fermented HB)~~ samples. Each duplicate of a time point sample was made in distinct 50 mL Falcon conical tubes (Corning, USA). Non inoculated autoclaved hemp bran (TBH) was used as control. Two biological replicates of each sample were performed. For each inoculated sample (C, L, M, and P) sampling was performed after 6, 24, 48, and 72 h as reported in Supplementary Table 1. Analyses were regarded to bacterial quantifications, pH, and VOCs ~~(volatile organic compounds)~~ characterization at least in duplicates.

2.4. Bacterial ~~CFU~~ Culture-Dependent Counting

For all bacteria 1 mL of each sample was aseptically transferred into a sterile tube ~~of~~ with 9 mL of physiological solution (0.9 g/dL NaCl) to be serially diluted (1/10) and plated in duplicates. Lactobacilli and the pool were counted on MRS agar (Oxoid, Thermo Fisher Scientific, USA) after incubation for at least 24 h at 37 °C in jars with anaerobiosis catalyst (Oxoid, Thermo Fischer Scientific, USA). *B. bifidum* NCIMB 700795 was counted on MRS agar supplemented with 0.005 g/dL L-cysteine (Sigma, USA) after incubation in the same conditions of lactobacilli. *E. coli* ATCC 25922 was counted on BHI agar (Oxoid, Thermo Fisher Scientific, USA) at 37 °C for 24 h.

2.5. pH measurement

The pH was determined with a pH-meter (Crison, Alella, Spain) at 20 °C appropriately calibrated with three standard buffer solutions at pH 9.21, pH 4.00, and pH 2.00. The pH values were measured in duplicate at three different times to monitor the fermentation.

2.6. Quantification by qPCR

Bacterial DNA from fermented hemp bran and from broths for prebiotic activity assay was extracted with the Pure Link Microbiome kit (Invitrogen, Thermo Fisher Scientific, USA). Genetic standards for qPCR were prepared from serially diluted PCR products (1/10) obtained amplifying gene targets with specific primers (Supplementary Table 2) with ProFlex PCR System (Thermo Fisher Scientific, USA) and SuperFi Platinum Taq (Thermo Fisher Scientific, USA), and purified with GeneJet PCR purification kit (Thermo Fisher Scientific, USA). qPCR was performed with a RotorGene 6000 (Qiagen, Hilden, Germany) and the RotorGene Q Series Software 2.3.1 (Qiagen, Germany). PCR and qPCR reactions were performed according to previously published protocols

(Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; Nissen, di Carlo & Gianotti, 2020; Nissen, Bordoni & Gianotti, 2020).

2.7. Prebiotic activity

The best performing time point, selected based on the best growth and pH reduction, was used to choose the FBH samples to screen for prebiotic activity, that was calculated with the related formula from two independent experiments and triplicates as previously described (Fissore, Santo Domingo, Gerschenson & Giannuzzi, 2015; Huebner, Wehling, Parkhurst & Hutkins, 2008), including qPCR quantifications (Nissen, di Carlo & Gianotti, 2020). FBH samples were filtered (Minisart® Syringe Filter 0.22 µm, Sartorius, Gottingen, Germany) and then all samples including BH and TBH controls were freeze dried using a Savant freeze-dryer Lyolab 3000 apparatus (Thermo Fisher Scientific, USA), in order to add a 1g/dL of product to 10 mL of culture media. FOS from chicory (Sigma, USA) was used as prebiotic positive control, and FH (commercial hemp seed flour) sample ~~was used~~ as an additional control~~, along with prebiotic positive control fructo-oligosaccharides (FOS) from chicory (Sigma, USA)~~. The media employed as control to calculate the prebiotic scores were instead added with 1g/dL of glucose. The bacterial type strains *Lp. plantarum*~~*Lb. plantarum*~~ 98b, *B. bifidum* NCIMB 700795, and *E. coli* ATCC 25922 were used at final concentration of 6 Log_{10} CFU/mL (Fissore, Santo Domingo, Gerschenson & Giannuzzi, 2015; Nissen, di Carlo & Gianotti, 2020).

2.8. Solid-Phase Microextraction Gas chromatography/Mass spectrometry (SPME-GC-MS)

Evaluation of VOCs was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV), equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, NL). The protocols for SPME-GC-MS analyses and for the identification of VOCs were previously published

(Guerzoni, Vernocchi, Ndagijimana, Gianotti & Lanciotti, 2007; Di Cagno et al., 2011; Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; ~~Nissen, di Carlo & Gianotti, 2020;~~ Nissen, ~~Casciano~~ di Carlo & Gianotti, 2020). Briefly, before each SPME sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. Prior analyses 6 µL of 10,000 mg/mk of 2-Pentanol, 4-methyl (Merck, Darmstadt, Germany) as internal standard were injected into the vial containing 3 mL of liquid sample and let to equilibrate for 10 min at 40 °C in a water bath. The SPME fiber was exposed to each sample for 40 min, and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 1 min, then programmed at 1.5 °C/min to 65 °C, and finally at 3.5 °C/min to 220 °C, which was maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in split-less mode and helium (3 mL/min) was used as a carrier gas. Identification was obtained with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). Acetic acid, Propionic acid, and Butyric acid were absolutely quantified in mg/kg ~~employing an internal standard~~ (Di Cagno et al., 2011; Nissen, di Carlo & Gianotti, 2020) (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg), while terpenes compounds were relatively quantified from chromatogram peak areas, as a ratio peak area/total peak of different samples (Bonfrate et al, 2020) (LOD = 0.001 mg/kg) and then normalized with the mean centering method (~~Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; Nissen, di Carlo & Gianotti, 2020;~~ Nissen, ~~Casciano~~ Bordoni & Gianotti, 2020). The samples analyzed were 3 mL of each time points case, namely 0 h, 6 h, 24 h, 48 h, and 72 h. The Samples analyzed were those collected from two technical replicas of two independent experiments.

2.9. Statistical analyses

All statistical analyses were performed using TIBCO Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA). Normality was checked with the Shapiro-Wilk's test and homoscedasticity was evaluated with the Levene's test (Granato, Araujo Calado & Jarvis, 2015). Differences between all samples

were evaluated with Analysis of Variance (ANOVA), while Principal Component Analysis (PCA), K-Means clustering, Spearman Rank Correlations, Two-way joining heatmap, and MANOVA were used to study the relationship between the variables (Nissen et al., 2020). To compare a sample to another within the same dependent variables a Student's T-test was employed ($P < 0.05$), while to compare different cases and different variables was used a Tukey's HSD (Honestly Significant Differences) test ($P < 0.05$). For PCA and Spearman Rank Correlations, the dataset was normalized using the mean centering method, including terpenes VOCs, delta pH, delta values of bacterial growth, and the prebiotic scores. All results are expressed as mean values obtained at least from duplicates batches in two independent experiments. qPCR and pH results were obtained from three replicates from two independent experiments.

3. Results

3.1. pH values, bacterial quantifications of TBH fermentations

pH values were expressed as delta reduction over time (Supplementary Table 3). ~~Starting from~~The initial pH of every sample had a ~~a~~ mean ~~pH~~ value of 6.55 ± 0.06, then acidification was induced ~~actively lasting~~ up to 24 h or ~~24 or~~ 48 h and a plateau was maintained afterwards. ~~After~~ Indeed, after 24 h, the mean pH reduction was 2.16 ± 0.19 ($P < 0.05$) ~~but~~ and no significant differences were seen up to the endpoint (72 h) ($P < 0.05$). C1112 (C) was the ~~best and fastest in the acidification of the medium, scoring the top value among the dataset at the early time point~~sample that after 24 h generated the maximum reduction of pH (-2.38 ± 0.17). Bacterial quantifications were expressed as means values of plate counts and qPCR results, and they were presented as cell number increase, expressed as Log_{10} cells/mL (Supplementary Table 4). Generally, in TBH samples all inocula kept growing exponentially up to 48 h (5.96 ± 0.31 Log_{10} cells/mL) ($P < 0.05$). M13 (M) and the bacterial pool (P) were the most competitive inocula, being faster and more long-lasting, for example at the endpoint the increases were about 6.35 ± 0.28 and 6.66 ± 0.35, respectively.

3.2. Volatile (low molecular weight) organic acids

Quantifications of volatile ~~acetic~~Acetic, ~~propionic~~Propionic, and ~~butyric~~ Butyric acids ~~i~~ares reported in mg/kg, and ~~the mean values of any~~ each mean value of F~~fermented HP~~BH samples ~~were~~ was compared to that of NF samples. In Figure 1A and Supplementary Table 5 it is described that the abundance of ~~acetic~~ Acetic acid increased on a time-basis. In ~~fact~~details, ~~it~~ this organic acid was found in traces (lower than 0.5 mg/kg) in NF cases but ~~after~~ fermentation of TBH by any bacterial inoculum was able to increase~~accounted for significantly higher means values~~ the content ($P < 0.05$). Samples C, M, and L (LB325) were able to ~~The~~increase the ~~trend of every sample fermented~~quantity of Acetic acid exponentially during fermentation up to the endpoint, while P ~~with single inoculum was defined by an exponential raise up to~~was able up to 48 h~~ours and followed by a lighter one up to the endpoint~~. ~~Differently acted the pool of strains whose curve reached earlier a higher top value and declined afterwards.~~ The maximum mean value ~~amid~~ among the dataset was that of P48, accounting for 11.13 ± 1.01 mg/kg, the double more than the mean of every single inoculum at that timepoint ($P < 0.05$). Considering the single ~~inocula~~inoculum, the best doer was C~~*Lb. rhamnosus* C1112~~ at 72 h (C72), recording 7.43 ± 0.72 mg/kg. The high levels of ~~acetate~~ Acetic acid recorded by P48 were consistent with high bacterial growth, but not with mild acidification observed.

In Figure 1B and Supplementary Table 5 the mean values of ~~propionic~~ Propionic acid are described. T~~For t~~his compound ~~, a similar scenario to acetic acid was seen. In fact, f~~from a very ~~low~~ little value in NF (0.04 ± 0.04 mg/kg) ~~, the production of propionic acid was raising~~raised constantly over time up to the endpoint for ~~the average of single inoculum~~C, L, and M, and up to 48 h for ~~the pool~~P. Considering the mean of values of C, L, and M, Propionic acid abundance was 5.75-, 10.58-, and 14.25-folds larger at 24, 48, and 72 h.~~Excluding not significant early time point increase ($P > 0.05$), the increment means of the single inocula were 5.75-, 10.58-, and 14.25-folds more at 24, 48, and 72 h, respectively.~~ Otherwise, the increment values performed by ~~the pool~~P were 24.00-, 29.00-, and 22.00-folds more at 24, 48, and 72 h, respectively. Thus, ~~the pool~~P already produced higher

yields at 24 h, 4.17-folds more than ~~the average of single inocula~~the mean of C, L, and M. The maximum value among the dataset was once more that of P48, scoring 1.16 ± 0.13 mg/kg, 2.74-folds more than the mean of every single inoculum at that timepoint ($P < 0.05$). Considering ~~the~~ single ~~inocula~~inoculum, the best performer was M~~*Lb. fermentum* MR13~~ at 72 ~~-~~h (M72), recording 0.71 ± 0.15 mg/kg.

Butyric acid quantification (Figure 1C and Supplementary Table 5) showed significant differences when ~~fermented cases~~ FBH samples were compared to NF ~~mean value~~samples ($P < 0.05$)~~), except for those at the early time point~~. ~~In fact, f~~From a very low value in NF (0.08 ± 0.02 mg/kg), ~~all single inoculum samples~~C, L, and M produced constant higher yields up to the endpoint, while ~~the pool~~P reached the top value at 48 h and declined slightly after. Excluding not significant early time point increase ($P > 0.05$), the increment means of ~~the single inocula~~C, L, and M were 5.00-, 11.58-, and 17.00-folds more at 24, 48, and 72 h, respectively. Otherwise the increases performed by ~~the pool~~P were 15.10-, 26.00-, and 20.08-folds more at 24, 48, and 72 h, respectively. Thus, ~~the pool~~P already produced higher yields at 24-h, 5.00-folds more than the ~~average~~ mean of single C, L, and M ~~single inocula~~. The maximum value among the dataset was once more that of P48, scoring 2.08 ± 0.17 mg/kg, 2.24-folds more than the mean of every single inoculum at that timepoint ($P < 0.05$). Considering the single inocula~~,~~, the best producer was C~~*Lb. rhamnosus* C1112~~ at 72 h (C72), recording 1.53 ± 0.14 mg/kg.

In summary, TBH samples fermented with the bacterial pool ~~accounted for~~recorded the highest ~~yields~~ values of the three organic acids~~, and the time of fermentation that gave overall the best performances was set~~, principally at 48 h.

3.3. Terpenes

Among the whole dataset of identified VOCs, we selected 37 compounds, based on their chemical class (terpenes and sesquiterpenes), normality distribution, significant difference of variance ($P <$ 0.05), and ~~renow~~proved~~n~~ bioactivity (Figure 2). From the PCAs (Figures ~~4A~~ 2A and 2B) a robust

plane was evidenced, based on two factors defining the 26.11% and 26.63% of total representations. Coupling PCAs to K-Means clustering analysis (Figure ~~4C~~2C) it was possible to identify five clusters of ~~samples~~ cases described by significant differences ($P < 0.05$) on ~~relative abundances of~~ 30 molecules. In Figure 2A, cluster 1 ~~(blue dot)~~ was positioned on quadrant III of PCA's plane oriented distant to the left side and grouped just NF samples. This cluster was described by 30 compounds but just eight had relative higher ~~($P < 0.05$)~~ abundances ($P < 0.05$) than FBH samples, such as β-~~pinene~~Pinene, γ-Elemene, cis-β-Farnesene, Aromadendrene, 9-~~methyldecalin~~Methyldecalin, α-Farnesene, Geraniol, and Myrtenal. Thereof, all other ~~samples~~ cases (n = 32) were relative to ~~the~~ FBH ~~ones~~ samples and were distributed in four specific clusters. Cluster 2 ~~(fuchsia dot)~~ included early time point~~all the sample~~cases~~s at the early time points plus two relatives to 24 h time point~~and other two (L24 and L24_2) ~~and was mainly fitted in quadrant IV of PCA's plane~~. This cluster was described by 17 variables, but just three ~~had abundancies relatively higher~~were more abundant than ~~those found in~~ other clusters ($P < 0.05$), *i.e.* ~~butylated~~ Butylated hydroxytoluene, trans-Pinocarveol, and *p*-Vinylguaiacol. Cluster 3 included ~~just MR13 and LB325 fermented samples~~FBH samples fermented by M and L at intermediate and end time points. ~~In particular, M13 at 24, 48, and 72 h time points, while LB325 at 48 and 72 h time points.~~ It was described by 24 compounds, but none ~~had abundancies~~was significantly higher than ~~those of~~ other clusters ($P > 0.05$). Cluster 4 included all FBH samples ~~the pool (P)~~ fermented by P ~~samples~~ except that at the early time point ~~and it was set in quadrant I of PCA's plane oriented to the top~~. It was described by 24 compounds, ~~whose~~ and eight ~~had abundancie~~were ~~s~~ significantly ~~higher~~ more abundant ($P < 0.05$) than those of other clusters, *i.e.* Caryophillene oxide, 1-(R)-α-Pinene, Eudesma-4(14),11-diene, *p*-Cymene, Myrcene, Δ-3-Carene, 1-Octen-3-ol, and Citronellol. Cluster 5 contained ~~all~~ the cases related to intermediate and end time point FBH samples fermented by C~~1112 fermented samples relatives to intermediate and end time points and was positioned in quadrant IV of PCA's plane~~. It was described by 23 terpenes ~~in varying abundancies, among which~~and in particular ~~that of~~ 4-Trimethylsilyl-9,9-dimethyl-9-silafluorene, 4(10)-Thujen-3-ol, acetate, and

Borneol were significantly more abundant ~~significantly higher ($P < 0.05$)~~ than ~~those of~~ other clusters ($P < 0.05$). In summary, the products of TBH fermentation that had the largest speciation and the highest ~~yield~~ abundance ~~of~~ in terpenes were ~~that~~ those obtained ~~relatively to TBH samples fermented by the pool and by~~by P and C~~1112~~ ~~at least~~ after 48 h ~~of incubation~~.

3.4. Targeted MANOVA: fermentation dynamics and strain performances

MANOVA ($P < 0.01$) was performed ~~The~~ on the d~~d~~ataset of the 37 normally distributed variables with two categorical predictors: i) ~~was categorized on~~the bacterial inoculum and ii) ~~on~~the time of fermentation ~~to perform MANOVA ($P < 0.01$)~~ (~~Figure 3A and B~~Tables 1 and 2), in order to address specifically the production of terpenes. Considering the different ~~fermenting agent~~bacterial inoculum (~~Figure 3A~~Table 1), 20 variables had significant differences ($P < 0.01$) and it emerged that ~~not fermented samples~~NF ~~(Control)~~samples were ~~the sole accountin~~described by~~g for~~ Geraniol (100%), ~~and for more than the 76.0% of total~~ 9-Methyldecalin (76.0%), and ~~72.1%~~ of β-Selinene (72.1%) ~~abundances~~. The quantities of the remaining 17 VOCs were all significantly augmented with fermentation~~. In particular, some compounds were produced in higher proportion by a given inoculum in respect to the others.~~ and differently by the inoculum. For example, C~~1112~~ was responsible for 48.9% of total production of α-Caryophillene, 40.6% of Borneol, and 43.2% of Eucalyptol. Fermentation by L~~B325~~ led to the production of 52.9% of total β-Linalool and 37.7% of total *p*-Cymen-8-ol. ~~Fermentation conducted by~~ M~~R13~~ was distinguished by 49.9% of total abundance of *p*-Cymene and by 50.5% of total Myrtenal. ~~The poo~~P~~l~~ was responsible for the 59.9% of the total yield in 1-(R)-α-~~pinene~~Pinene, for the 63.9% of total γ-Elemene, the 57.6% of total cis-β-Farnesene, the 51.8% of total Myrcene, the 44.8% of total Δ-3-~~carene~~Carene, the 47.8% of total Fenchyl alcohol, the 45.8% of total 4(10)-Thujen-3-ol, acetate, and the 45.8% of total Eugenol. So far, TBH fermented by ~~the pool showed to be the inoculum that accounted for higher production of more compounds than the single strains. In fact, the pool~~P ~~produced~~ had higher amount of 9 terpenes in comparison to the 3 of C~~1112~~, and the 2 of both L~~B325~~ and M~~R13~~.

~~Instead, considering the~~ MANOVA categorized for the time points of fermentation showed that~~,~~ 20 ~~compounds~~ VOCs had significant differences ~~among the independent variables~~ ($P < 0.01$) (~~Figure~~ Table 2~~3B~~). Seven terpenes were intrinsic features of HPB and were not subject to significant increases ~~through~~ with fermentation, in particular~~.~~ β-Pinene, ~~and~~ Geraniol, and α-Farnesene ~~was~~ were ~~almost not detected after fermentation~~an exclusive signature of NF samples. ~~The other compounds were: Aromadendrene, accounting for the 66.4% of total abundance, 9-Methyldecalin, accounting for the 76.0%, β-Selinene for the 72.1%, and α-Farnesene for the 94.6%.~~ For any other compound, the abundance was in higher proportion at the late time points. ~~In fact~~In brief, ~~, after 24 h just the 33.7% of total Eucalyptol and the 62.6% of total p-Vinylguaiacol were produced. Instead, after~~at 48 h the VOCs discriminated were: ~~70.9% of total~~ Eudesma-4(14), 11-diene~~,~~ (70.9%), ~~as well as the 50.9% of total~~ *p*-Cymene (50.9%), ~~the 56.6% of total~~ Terpinolene (56.6%), ~~the 40.6% of total~~ 1-Octen-3-ol (40.6%), and ~~the 42.1% of total~~ 2-Decen-1-ol, (E) (42.1%)~~were produced~~. Lastly, at the end point major proportions on total yields of γ-Elemene (84.4%), of Citronellol (38.6%), and of Myrtenal (54.4%) were achieved.

3.5. Prebiotic score

The prebiotic scores were calculated from the equation proposed by Huebner, Wehling & Hutkins, (2007) and revised by Fissore, Santo Domingo, Gerschenson & Giannuzzi, (2015), which considers the effect of a fiber in comparison to glucose towards the growth of a beneficial bacteria in respect to the growth of pathogenic *E. coli*. The highest score for prebiotic activity (Table ~~1~~3) versus *Lp. plantarum* ~~*Lb. plantarum*~~ 98b was achieved by P48, that was the sole sample scoring significantly higher than FOS ($P < 0.05$). In ~~fact~~particular, TBH fermented by P48 was significantly stronger than FOS in the containment of *E. coli* ATCC 25922 ~~even if the growth of *Lb. plantarum* 98b on 1 g/dL of TBH fermented by P48 was slightly lower than that of FOS, the inhibition of the former on *E. coli* ATCC 25922 was significantly stronger~~ ($P < 0.05$) (Supplementary Table 6). Besides, in comparison to FH, TBH fermented by P48~~P48 reached a prebiotic score on~~had a prebiotic score

1.8-folds higher versus *Lp. plantarum* 98b ~~*Lb. plantarum* 98b 1.8-folds higher than FH that had the lowest value~~. The prebiotic score raised in respect to~~score raised in respect to~~ the intensity of HPB treatment, in details BH had the lowest value and TBH the highest~~from the lowest of BH to the top of fermented TBH~~. Among the fermented samples the runner up was C48, with a score slightly lower than FOS, but significantly higher than similar samples (L48 and M48). Considering the prebiotic activity towards *B. bifidum* NCIMB 700795 (Table ~~1~~3), ~~a similar trend was evidenced. T~~the best performing sample was C48, higher than FOS and P48, but with no significant difference ($P > 0.05$). ~~Even in this context,~~Similarly to the previous prebiotic target, FOS ~~made~~ foster more *B. bifidum* NCIMB 700795 ~~to grow more~~ than the best TBH fermented sample (C48), but this latter was stronger in the containment of *E. coli* ATCC 25922 ~~inhibition~~ ($P < 0.05$) (Supplementary Table 6). Besides, TBH fermented by C48 hit the top prebiotic score versus *B. bifidum* NCIMB 700795 and was ~~S~~significantly different from any other samples ($P < 0.05$)~~ces were seen in respect to all other samples. and in particular the prebiotic activity of C48 was 1.7 folds more effective than that of FH. Thus, both C48 and then P48 scored higher values than other fermented or not fermented samples ($P < 0.05$).~~ In brief, ~~among the strains tested~~ after 48 h of fermentation of ~~the pool~~TBH, the pool demonstrated to produce a substrate ~~that had the top~~with the best prebiotic activity versus ~~*lactobacilli*~~*Lp. plantarum*, while C~~1112~~ ~~hit the top~~with the best versus *B. bifidum* ~~bifidobacteria~~.

3.6. Spearman rank correlations

We used Spearman rank analysis to evidence ~~d~~correlations between variables related to ecological features (bacterial growth, pH decrease, and prebiotic activity) and those related to abundance~~s~~ of VOCs ~~considered~~, on independent variables (n = 32) (Figure 43). ~~Considering the bacterial growth, t~~The variable "delta cells" indicates the ~~was obtained from the~~ difference ~~of~~in Log_{10} cells/mL ~~at the endpoint and~~between ~~the~~ beginning and end of fermentation. Significant correlations ($P < 0.05$) ~~indicated~~ evidenced that during fermentation of TBH samples the ~~the more~~ growth of bacteria~~l grew in fermentation the more~~was positively correlated with quantity ~~of~~ SCFAs, ~~and minorly~~ *p*-

Cymene, and Citronellol ~~was found in fermented TBH samples~~. In contrast three terpenes, *i.e.* Caryophyllene, Δ-3-Carene, and β-Selinene, were inversely ~~proportional~~ correlated to bacterial growth. It is likely that their accumulation in the substrate resulted in a constraint for lactobacilli. As a matter of fact,~~Considering that~~ from previous MANOVA ($P < 0.05$) resulted that the longer was the fermentation time the larger the quantity of these three VOCs~~from MANOVA was found that almost 50% of total yield of these VOCs was fostered by fermentation., it is likely that their accumulation in the substrate resulted in a constraint for lactobacilli as the fermentation was prolonged over time when bacterial load was richer.~~

~~Acetic acid abundance was even significantly proportional to acidification, as well as that of 1-Octen-3-ol and Fenchyl alcohol.~~ Considering the prebiotic activity, it is interest to stress out that correlation trend was similar for both probiotics. A group of ~~terpenes~~ VOCs including *p*-Cymene, Myrcene, Eugenol, 1-Octen-3-ol, Terpinolene, and β-Pinene resulted significantly associated to prebiotic activity ($P < 0.05$), while Caryophyllene, Eucalyptol, and β-~~linalool~~ Linalool were negative~~inverse~~ly ~~proportional~~ correlated ($P < 0.05$). This issue could mean that just the ~~former list of~~ VOCs related to prebiotic activity ~~has~~ generate ~~a~~ selective ~~bioactivity versus certain bacteria~~effects, *e.g.* ~~inhibiting~~ inhibition of enteropathogenic *E. coli* ~~enteropathogens~~ and capacity to foster~~ing~~ ~~*probiotics*~~ *B. bifidum* or ~~*lactobacilli*~~*Lp. plantarum*~~, instead the latter list of VOCs had a broader spectrum of antimicrobial activity~~.

4. Discussion

~~When TBH was fermented, interestingly~~ Interestingly, when TBH was fermented the high lactobacilli ~~load~~growth~~,~~ and the high content of ~~acetic~~ Acetic acid did not lead to extreme acidification levels. In fact, the pH values were not reduced excessively (C1112 hit top acidification after 48 h with pH value of 4.21 ± 0.02), ~~likely~~ like happens ~~during the most of fermentation processes performed on~~when plant-based material are fermented. For example, lactobacilli fermenting carrot, cabbage or radish can bring pH down to less than 4 after 72 h (Vatansever, Vegi,

Garden-Robinson & Hall, 2017). Kimchi fermentation by indigenous LAB~~, including *Lactobacillus spp.*~~, can acidify the substrate up to pH 3.5 after 24 h (Joon-Yeon & Kunz, 2009). In our work, from 24 h to the end~~time~~ point the pH ~~remained~~ was stable with a mean value of 4.41 ± 0.15~~, besides~~ and ~~in this period~~ bacterial cells kept growing up to ~~an endpoint~~a mean of 12.23 ± 0.24 Log_{10} cells/mL. Therefore, HPB positively ~~reflects the essential~~showed ~~characteristic of a~~to be a substrate ~~for~~ which fosters probiotic growth ~~including~~ and owns a pH buffering capacity (Nissen, di Carlo & Gianotti, 2020).

The prebiotic activity ~~scored~~ recorded by fermented TBH was surprisingly effective even due to a stronger containment of the growth of *E. coli* ATCC 25922, in comparison to other samples and the FOS. Hemp female inflorescences and hemp seeds bring many terpenes with renowned antimicrobial activity~~(Nissen et al., 2010~~ (Pellati, ~~Brighenti, Sperlea, Marchetti, Bertelli & Benvenuti~~et al., 2018; Leghissa, Hildenbrand & Schug, 2018), that alone or in synergism show to be capable to inhibit opportunistic and food borne pathogens (Nuutinen, 2018; Nafis et al., 2019). ~~The antimicrobial activity of hemp seed is reckoned to be triggered by the synergistic effects of different terpenes present in hemp seed oil (Nafis et al., 2019, Nissen et al., 2010).~~ On the contrary, other plant-based materials able to foster probiotics do not have a prebiotic activity because cannot tackle the growth of enteropathogens (Vieira, Bedani, Albuquerque, Biscola & Saad, 2017). A fundamental criterium to classify a food ingredient as a prebiotic is the scientific ability to foster the growth and support the activity of beneficial intestinal bacteria (Gibson et al. 2017). In this view the assay of prebiotic activity adopted reflects the ability of a prebiotic to ~~jointly~~mutually foster the growth of probiotics and limit that of enteropathogens in comparison to glucose (Fissore, Santo Domingo, Gerschenson & Giannuzzi, 2015; Huebner, Wehling, Parkhurst & Hutkins, 2008). Another element that supports the ~~The s~~strong prebiotic activity of FBH that we have observed could be ~~partly due to~~ the ~~recorded~~ high~~er~~ quantity~~levels~~ of ~~acetic~~Acetic, ~~propionic~~Propionic, and ~~butyric~~ Butyric acids generated ~~s, particularly~~ by fermentation with ~~in~~ P48 and ~~in~~ C72. Lactobacilli are able to liberate and produce low organic acids during fibers

degradation, thus improving the original content in the fermented product (Massa et al., 2020). The quality and quantity of these organic acids depend on the type of fiber used (Gill, van Zelm, Muir & Gibson 2018).

~~In fact~~T~~, t~~he beneficial effects of low organic acids are renowned ~~and~~ , ~~are multi-targets, not solely directed to the host epithelial mucosa, and to the blood stream, but even to the microbiota, as a selective substrate (Goverse et al., 2017). A~~abundant production of these compounds is linked to well-being (Goverse et al., 2017) and their nutritional supplementation ~~are~~ is suggested for the treatment of ~~in~~ different intestinal diseases (Gill, van Zelm, Muir & Gibson 2018).

From Spearman Rank correlation, the abundance of *p*-Cymene, Myrcene, Eugenol, 1-Octen-3-ol, Terpinolene, and β-Pinene was linked to prebiotic activity, and all these VOCs, except the latter, were increased with fermentation of TBH. These results are in line with recent literature, where it is reported that bacterial fermentation is able to improve the original terpenes content of cheese (Alves Bezerra et al., 2017), mango peel (Jin et al., 2018), okara from soybean (Gupta, Lee & Chen, 2018), hemp seed drinks (Nissen, di Carlo & Gianotti, 2020), hemp seed enriched doughs (Nissen, Bordoni, Gianotti, 2020), and blueberry pomace (Cheng et al., 2020). The antimicrobial properties and the applications in foods of terpenes such as *p*-Cymene (Marchese et al., 2017), Terpinolene (Fiorini et al., 2019; Karas et al., 2020), Myrcene (Mitropoulou et al., 2107), and Eugenol (Talón, Vargas, Chiralt & González-Martínez. 2019) is proved. Additionally, these VOCs are promising health related compounds due to the strong anti-oxidant and anti-inflammatory capacity, as good radicals' scavengers (De Oliveira et al., 2015; Boulebd, 2021; Yi, Sun, Bao, Ma & Sun, 2019; da Silva et al., 2018).~~Myrcene that was plenty in FBH samples. In fact, this monoterpene rules almost a third of Futura 75 hemp seed essential oil content (Nissen et al., 2010), and besides has antioxidant and anti-inflammatory capacities (Mitropoulou et al., 2107; Yi, Sun, Bao, Ma & Sun, 2019). Then we found high abundance of Terpinolene, that is a monoterpene and a structural isomer of (+)-Limonene that is found largely in hemp inflorescence up to 9.7% of total weight, and is currently used in the food industry (Fiorini, Molle, Nabissi, Santini, Benelli & Maggi, 2019). It has~~

been reported to have antioxidant capacities as a good radicals' scavengers (Boulebd, 2021), and to act as antimicrobial (Karas, Wong, Paulin, Mazeh, Hussein, Li & Velkov, 2020). Eugenol is a natural phenolic compound found abundantly in cinnamon and in clove essential oils and is the main responsible for clove aroma (Talón, Vargas, Chiralt & González-Martínez, 2019). Eugenol is indicated for several therapeutic effects, because is a good radicals' scavengers with antibacterial effect (da Silva, Monte, de Lemos, do Nascimento, Costa, de Paiva, 2018). *p*-Cymene is a monoterpene found in more than 100 plant species able to counteract different food borne pathogens (Marchese et al., 2017). It shows numerous biological activities, increasing the activity of antioxidant enzymes, contributing to reduce oxidative stress (De Oliveira et al., 2015).

Thus, according to the new definition of prebiotics, FBH had an higher prebiotic score than FOS because its effect was jointly generated by other beneficial compounds and the polysaccharides. In fact, considering metabolomics, multivariate analysis defined that HPB samples prior fermentation were described by 28 different terpenes, whose two were exclusively found at this stage, such as β-Pinene and Geraniol. The other compounds were all positively subjected to the effect of fermentation process, which have surged their release. Consequently, in our study the more the terpenes bioactive VOCs (low organic acids and terpenes) were abundant and the more the prebiotic activity was effective. , microbial fermentation is able to improve the original terpenes content of cheese (Alves Bezerra et al., 2017), mango peel (Jin et al., 2018), okara from soybean (Gupta, Lee & Chen, 2018), hemp seed drinks, and hemp seed enriched doughs (Nissen, di Carlo, Gianotti, 2020; Nissen, Bordoni, Gianotti, 2020), and blueberry pomace (Cheng et al., 2020). Actually, from Spearman Rank correlation, the abundance of *p*-Cymene, Myrcene, Eugenol, 1-Octen-3-ol, Terpinolene, and β-Pinene was linked to prebiotic activity, and all these VOCs, except the latter, were increased throughout fermentation of TBH.

Thus, according to the new definition of prebiotics, FBH had a higher prebiotic score than FOS because its effect was jointly generated by other beneficial compounds and the polysaccharides.

Lactobacilli are able to liberate and produce low organic acids during fibers degradation, thus improving the original content in the fermented product (Massa et al., 2020). Besides the organic acids production is differently triggered by different fibers (Gill, van Zelm, Muir & Gibson 2018). Similarly, microbial fermentation is able to improve the original terpenes content of cheese (Alves Bezerra et al., 2017), mango peel (Jin et al., 2018), okara from soybean (Gupta, Lee & Chen, 2018), hemp seed drinks, and hemp seed enriched doughs (Nissen, di Carlo, Gianotti, 2020; Nissen, Bordoni, Gianotti, 2020), and blueberry pomace (Cheng et al., 2020).

Hemp female inflorescences bring many terpenes (Nissen et al., 2010; Pellati, Brighenti, Sperlea, Marchetti, Bertelli & Benvenuti, 2018; Leghissa, Hildenbrand & Schug, 2018), that alone or in synergism show to be capable to inhibit food borne pathogens (Nafis et al., 2019).

Among the most effective terpenes that we have detected and described there were five that recently attracted scientist for their biological activities, namely Myrcene, Terpinolene, Eugenol, p-Cymene, and 1-Octen-3-ol. The first is Myrcene that was plenty in FBH samples. In fact, this monoterpene rules almost a third of Futura 75 hemp seed essential oil content (Nissen et al., 2010), and besides has antioxidant and anti-inflammatory capacities (Mitropoulou et al., 2107; Yi, Sun , Bao, Ma & Sun, 2019). Then we found high abundance of Terpinolene, that is a monoterpene and a structural isomer of (+) Limonene that is found largely in hemp inflorescence up to 9.7% of total weight, and is currently used in the food industry (Fiorini, Molle, Nabissi, Santini, Benelli & Maggi, 2019). It has been reported to have antioxidant capacities as a good radicals' scavengers (Boulebd, 2021), and to act as antimicrobial (Karas, Wong, Paulin, Mazeh, Hussein, Li & Velkov, 2020). Eugenol is a natural phenolic compound found abundantly in cinnamon and in clove essential oils and is the main responsible for clove aroma (Talón, Vargas, Chiralt & González-Martínez, 2019). Eugenol is indicated for several therapeutic effects, because is a good radicals' scavengers with antibacterial effect (da Silva, Monte, de Lemos, do Nascimento, Costa, de Paiva, 2018). *p*-Cymene is a monoterpene found in more than 100 plant species able to counteract different food borne

~~pathogens (Marchese et al., 2017). It shows numerous biological activities, increasing the activity of antioxidant enzymes, contributing to reduce oxidative stress (De Oliveira et al., 2015).~~

~~1-Octen-3-ol has been described in different plants and fungi and is a short- chain oxylipin, oxygenated derivative of linoleic acids, able to protect plants in response to external challenges (Zhang et al., 2021) and successfully tested *in vitro* against food borne pathogens (Xiong, Li, Li, Chen, Chen, Huang, 2017).~~

~~The bioactives that we have described use to act in synergism, resulting to exalt the beneficial effect for the host (Nafis et al., 2019; Nissen et al., 2010; Russo, 2011); for example, it has been proposed that phytocannabinoids and terpenoids interact together to deliver joint effect in the treatment of inflammation and microbial pathogenesis (Nuutinen, 2018).~~

~~In this way a human colonic model may represent the proper *in vitro* approach for a deep knowledge of potential functionalities of HPB (Nissen, Casciano, Chiarello, Di Nunzio, Bordoni & Gianotti, 2021). Throughout the aid of such tools it will be possible to landscape the complexity of the shift of the microbial populations, as restraint of enteropathogens and promotion of probiotics, and to reveal in detail the metabolic faith of bioactive VOCs.~~

5. Conclusions

Development of functional foods for our next future could be afforded just employing sustainable approaches and renewable feedstock, including the exploitation of industrial byproducts. These needs could be satisfied by hemp seed that is a multi-tasking feedstock, is a powerhouse of nutrients and health related compounds. However~~Its cultivation rinses the soil and gives generous yields with low-demanding attributes in terms of chemicals, land, and water. Also~~, the industrial process uses to discard potentially high value plant component, such as ~~hemp seed bran~~HPB, that deserves to be valorized.

In the present work we have demonstrated that fermented hemp seed bran could be considered ~~technically improved with fermentation, resulting in~~as a product with higher prebiotic activity due

to more ~~is possible to extend to the byproducts the nutritional and health potential characters of the principal feedstock, as well as to increase the original content and yields of certain~~ bioactives, such as ~~SCFA~~ organic acids which fostered the growth of probiotics, and ~~some selected~~ terpenes that tackled enteropathogenic *E. coli*.

This work discovered that HPB is a fine substrate for beneficial lactobacilli ~~, as well as for probiotics~~and propose to apply HPB as a prebiotic ingredient~~. Furthermore, for the first time, unprocessed or differently treated and fermented HPB were assessed, compared, and discussed on their prebiotic potential, targeting also the VOCs deputed to this bioactivity~~. Besides, with fermentation of HPB the bioactives related to prebiotic activity could be increased. We have found that the prebiotic activity of HPB samples is the result of the action of a pool of health-related compounds, and that their presence is related to~~production could be balanced by fermenta~~ fermentation process~~tion~~ with specific bacteria~~ Considering the synergistic effort that different terpenes generally use for the generation of the final bioactive effect, we can write that even the prebiotic activity of HPB samples is the result of the action of a pool of health-related compounds~~. ~~The results were obtained by a robust multivariate statistical approach that permitted to discriminate the contribution of each inoculum fermenting HPB on the prebiotic properties of the final product.~~ Although this work is still an *in vitro* investigation, it ~~The present research~~ offers some statistically robust basics over the valorization of an outcasted byproduct derived from the industrial transformation chain of hemp seed foods ~~and the recipient results vow that~~and introduces that HPB could have prebiotic application.

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Credit authorship contribution statement

Lorenzo Nissen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Flavia Casciano: Formal analysis, Investigation, Writing - review & editing.

Elena Babini: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing - review & editing.

Andrea Gianotti: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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.

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Figure captions

Figure 1. A) Acetic acid, B) Propionic acid, and C) Butyric acid quantification by SPME GC-MS of not fermented (NF) and fermented hemp bran (FBH), expressed in mg/kg. Plots are indicating results from two different replications and two independent experiments. Boxes indicate means values; rectangles indicate means values ± S.D.; whiskers indicate means values ± 1.96*S.D. NF = not fermented TBH; C6, C24, C48, and C72 = TBH fermented by *Lacticaseibacillus rhamnosus* C1112 after 6 h, 24 h, 48 h, and 72 h; L6, L24, L48, and L72 = TBH fermented by *Lactiplantibacillus plantarum subsp. plantarum* LB325 after 6 h, 24 h, 48 h, and 72 h; M6, M24, M48, and M72 = TBH fermented by *Limosilactobacillus fermentum* MR13 after 6 h, 24 h, 48 h, and 72 h; P6, P24, P48, and P72 = TBH fermented by the pool after 6 h, 24 h, 48 h, and 72 h. Means with different letters are significantly different at $P < 0.05$ by Student T-test.

Figure 2. Multivariate analysis on 37 VOCs terpenes quantified by SPME GC-MS of not fermented (NF) and fermented hemp bran (FBH) samples. (A) PCA of cases; (B) PCA of variables; (C) K-Means clusterization based on dependent variables. Cluster 1 = blue plot; Cluster 2 = fuchsia plot; Cluster 3 = green plot; Cluster 4 = black plot; Cluster 5 = yellow plot. *1,4,7, Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-; **4-Trimethylsilyl-9,9-dimethyl-9-silafluorene; ***Phenol, 2,4-bis(1,1-dimethylethyl)-. Codes of samples: X0 = not fermented samples; C6, C24, C48, and C72 = TBH fermented by *Lacticaseibacillus rhamnosus* C1112 after 6 h, 24 h, 48 h, and 72 h; L6, L24, L48, and L72 = TBH fermented by *Lactiplantibacillus plantarum subsp. plantarum* LB325 after 6 h, 24 h, 48 h, and 72 h; M6, M24, M48, and M72 = TBH fermented by *Limosilactobacillus fermentum*

MR13 after 6 h, 24 h, 48 h, and 72 h; P6, P24, P48, and P72 = TBH fermented by the pool after 6 h, 24 h, 48 h, and 72 h.

~~Figure 3. MANOVA plots of terpenes with categorical predictors ($P < 0.01$) set on inocula (A) and on time (B). ***Phenol, 2,4-bis(1,1-dimethylethyl) . % values indicate the contribution of each categorized cases on the total load on the dataset of each dependent variable (the VOCs)~~

Figure ~~4~~3. Two-way joining heatmap of double matrix Spearman rank correlations on VOCs terpenes of 32 independent variables from treated hemp bran (TBH), including not fermented TBH and TBH fermented (FBH) for 6 h, 24 h, 48 h, and 72h at 37 °C by *Lacticaseibacillus rhamnosus* C1112, *Lactiplantibacillus plantarum subsp. plantarum* LB325, *Limosilactobacillus fermentum* MR13, and by the pool of these three strains. * $P < 0.05$. X Axis labels: Delta cells = Log cells/ml increase; [Delta pH] = Acidification of substrate; PS *Lb. plan* = Prebiotic Score on *Lactiplantibacillus plantarum subsp. plantarum* 98b; PS *B. bif* = Prebiotic Score on *Bifidobacterium bifidum* NCIMB 700795.

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Table 1. MANOVA based on bacterial inoculum as categorical predictor. % of production and significance.

VOC	NF	C1112	LB325	MR13	Pool	P value
α-Caryophyllene	11.24^{c}	48.85^{a}	6.54^{d}	6.74^{d}	26.63^{b}	0.00605
1R-α-Pinene	15.33^{b}	7.97^{bc}	10.80^{b}	5.94^{c}	59.96^{a}	0.00121
p-Cymene	13.20^{c}	0.00^{e}	4.56^{d}	49.88^{a}	32.36^{b}	0.00955
γ-Elemene	7.53^{c}	22.67^{b}	5.82^{c}	0.00^{d}	63.98^{a}	0.01410
cis-β-Farnesene	5.17^{c}	18.60^{b}	18.60^{b}	0.00^{d}	57.63^{a}	0.00850
Myrcene	19.53^{b}	11.78^{c}	0.00^{d}	16.90^{b}	51.79^{a}	0.00788
Δ-3-Carene	5.68^{c}	22.92^{b}	22.24^{b}	4.31^{c}	44.84^{a}	0.04027
9-Methyldecalin	76.05^{a}	6.95^{b}	9.15^{b}	0.00^{c}	7.86^{b}	0.00012
β-Selinene	72.15^{a}	3.89^{c}	6.04^{c}	0.00^{d}	17.92^{b}	0.00324
Geraniol	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	<0.00001
β-Linalool	4.94^{d}	20.76^{b}	52.94^{a}	4.81^{d}	16.55^{c}	0.00002
p-Cymen-8-ol	7.70^{c}	23.33^{b}	37.75^{a}	8.13^{c}	23.09^{b}	0.00954
Fenchyl alcohol	0.00^{c}	19.14^{b}	16.56^{b}	16.49^{b}	47.81^{a}	0.00103
4(10)-Thujen-3-ol, acetate	6.04^{d}	29.67^{b}	10.98^{c}	7.49^{cd}	45.82^{a}	0.00006
Borneol	2.10^{e}	40.56^{a}	16.63^{c}	7.81^{d}	32.89^{b}	0.00285
Eucalyptol	6.48^{cd}	43.21^{a}	21.07^{b}	10.30^{cd}	18.93^{b}	0.00522
Eugenol	36.63^{a}	2.23^{d}	15.34^{c}	0.00^{e}	45.81^{a}	0.00314
Phenol, 2,4-bis***	39.12^{a}	7.59^{d}	16.84^{c}	9.65^{d}	26.80^{b}	0.00165
Citral	6.18^{c}	25.87^{a}	15.41^{b}	27.07^{a}	25.47^{a}	0.00160
Myrtenal	1.76^{d}	2.23^{d}	9.15^{c}	50.50^{a}	36.36^{b}	0.00408

**Phenol, 2,4-bis(1,1-dimethylethyl)-; ^{abc}Different letters indicate statistical significance by Tukey's HSD post-hoc test ($P < 0.05$); NF = Not Fermented TBH; C1112 = TBH fermented by *Lacticaseibacillus rhamnosus* C1112; LB325 = TBH fermented by *Lactiplantibacillus plantarum subsp. plantarum* LB325; MR13 = TBH fermented by *Limosilactobacillus fermentum* MR13; Pool = TBH fermented by bacterial pool.

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Table 2. MANOVA based on time of fermentation as categorical predictor. % of production and significance.

VOC	0 h*	6 h	24 h	48 h	72 h	P value
β-Pinene	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	<0.00001
Eudesma-4(14), 11-diene	2.42^{c}	18.42^{b}	0.00^{c}	70.92^{a}	8.24^{b}	0.01642
p-Cymene	20.47^{b}	0.00^{c}	16.73^{b}	50.99^{a}	11.81^{b}	0.01179
γ-Elemene	10.18^{b}	2.22^{c}	1.46^{c}	1.69^{c}	84.45^{a}	0.04626
Terpinolene	6.16^{b}	33.44^{a}	3.70^{b}	56.64^{a}	0.06^{b}	0.04962
γ-Terpinene	45.10^{a}	16.95^{b}	4.30^{c}	17.81^{b}	15.84^{b}	0.04564
Aromadendrene	66.43^{a}	33.57^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00569
9-Methyldecalin	76.05^{a}	14.73^{b}	6.98^{bc}	2.24^{bc}	0.00^{c}	0.00006
β-Selinene	72.15^{a}	6.87^{bc}	17.09^{b}	3.89^{c}	0.00^{c}	0.00397
α-Farnesene	94.64^{a}	2.59^{b}	2.04^{b}	0.73^{b}	0.00^{b}	0.00006
Geraniol	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	<0.00001
1-Octen-3-ol	10.43^{bc}	6.72^{c}	23.68^{abc}	40.64^{a}	18.54^{abc}	0.01441
Citronellol	9.36^{b}	6.65^{b}	7.88^{b}	37.50^{a}	38.61^{a}	0.04779
2-Decen-1-ol, (E)-	6.43^{c}	8.33^{bc}	32.69 ^{ab}	42.09^{a}	10.47^{bc}	0.00332
Fenchyl alcohol	0.00^{b}	22.61^{a}	27.35^{a}	26.66^{a}	23.38^{a}	0.00005
Eucalyptol	7.93^{b}	22.48^{ab}	33.68^{a}	13.71^{ab}	22.20^{ab}	0.00163
Eugenol	64.26^{a}	0.00^{c}	1.08^{c}	13.05^{b}	22.71^{b}	0.00091
trans-Pinocarveol	0.00^{c}	27.10^{a}	16.40^{ab}	34.71^{a}	21.79^{a}	0.03245
p-Vinylguaiacol	0.00^{b}	5.18^{b}	62.58^{a}	27.71^{ab}	4.54^{b}	0.04211
Myrtenal	4.18^{b}	7.78^{b}	17.21^{b}	16.48^{b}	54.35^{a}	0.04909

^{abc}Different letters indicate statistical significance by Tukey's HSD post-hoc test ($P < 0.05$); *hours of fermentation.

Table 3 R2
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Table 1. Prebiotic activity scores obtained from cell density (Log_{10} cells/ml) of bacterial cultures grown with 1 g/dL of differently treated hemp seed bran as carbohydrate sources.

Type	**score on *Lactiplantibacillus plantarum subsp. plantarum* 98b**	**score on *Bifidobacterium bifidum* NCIMB 700795**
P48	0.408 ± 0.077^{d}	0.259 ± 0.043^{c}
C48	0.359 ± 0.088^{c}	0.307 ± 0.033^{c}
L48	0.279 ± 0.096^{b}	0.201 ± 0.052^{b}
M48	0.252 ± 0.039^{b}	0.226 ± 0.029^{b}
TBH	0.308 ± 0.071^{c}	0.243 ± 0.027^{b}
BH	0.258 ± 0.084^{b}	0.234 ± 0.046^{b}
FH	0.227 ± 0.079^{b}	0.182 ± 0.028^{b}
FOS	0.362 ± 0.042^{c}	0.305 ± 0.031^{c}

Values are means of three different replications and two independent experiments ± standard deviation. ^{a,b,c,d} = means with different letters within a column are significantly different at $P < 0.05$ by Student T-test. P48 = 48 h bacterial pool fermented, filtered, and freeze-dried hemp seed bran; C48 = 48 h *Lacticaseibacillus rhamnosus* C1112 fermented, filtered, and freeze-dried hemp seed bran; L48 = 48 h *Lactiplantibacillus plantarum subsp. plantarum* LB325 fermented, filtered, and freeze-dried hemp seed bran; M48 = 48 h *Limosilactobacillus fermentum* MR13 fermented, filtered, and freeze-dried hemp seed bran; TBH = thermally treated and freeze-dried hemp seed bran; BH = hemp seed bran; FH = hemp seed flour; FOS = fructooligosaccharides from chicory.

Figure 1 R2

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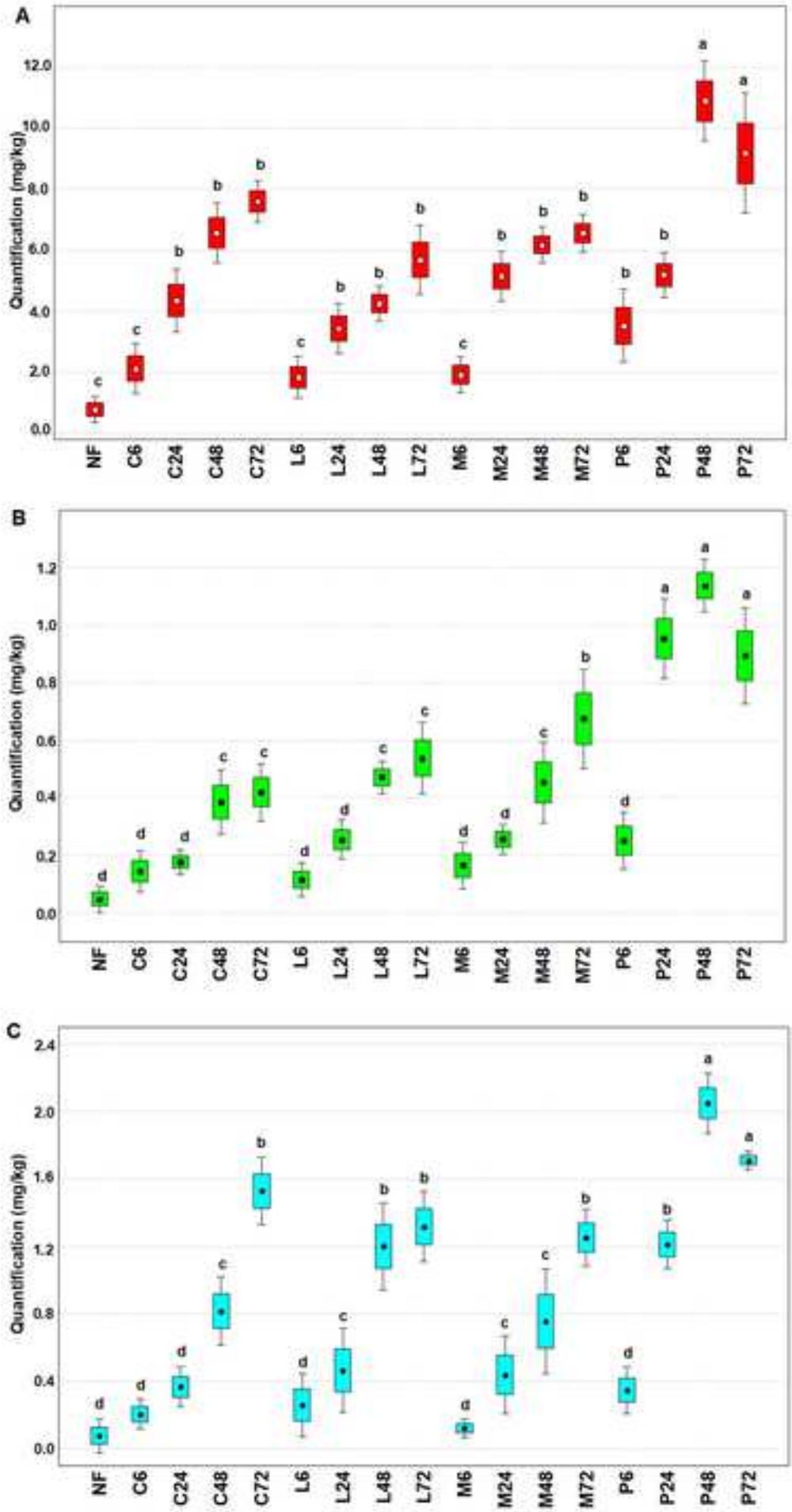

Figure 2

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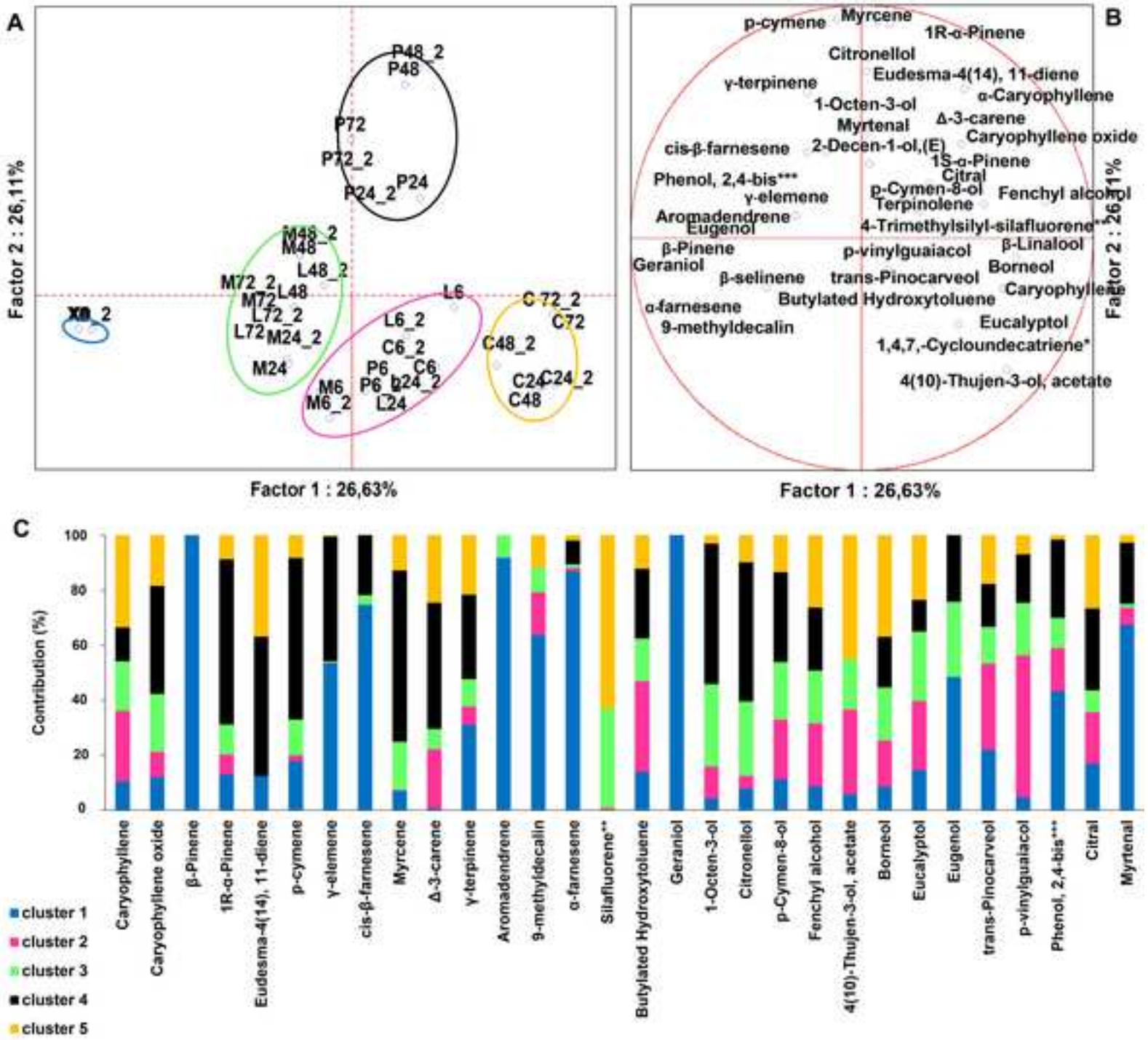

Figure 3 R2

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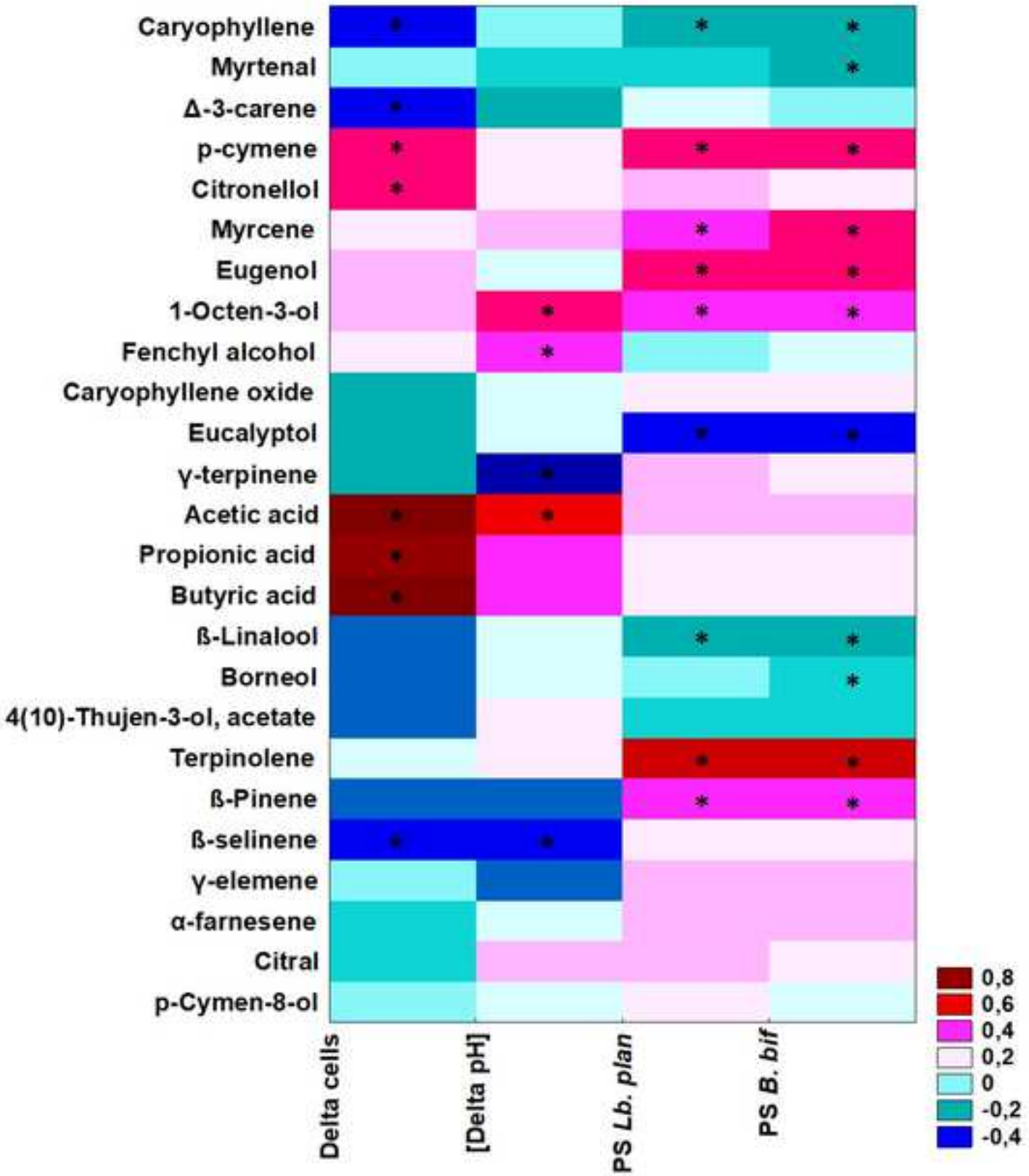

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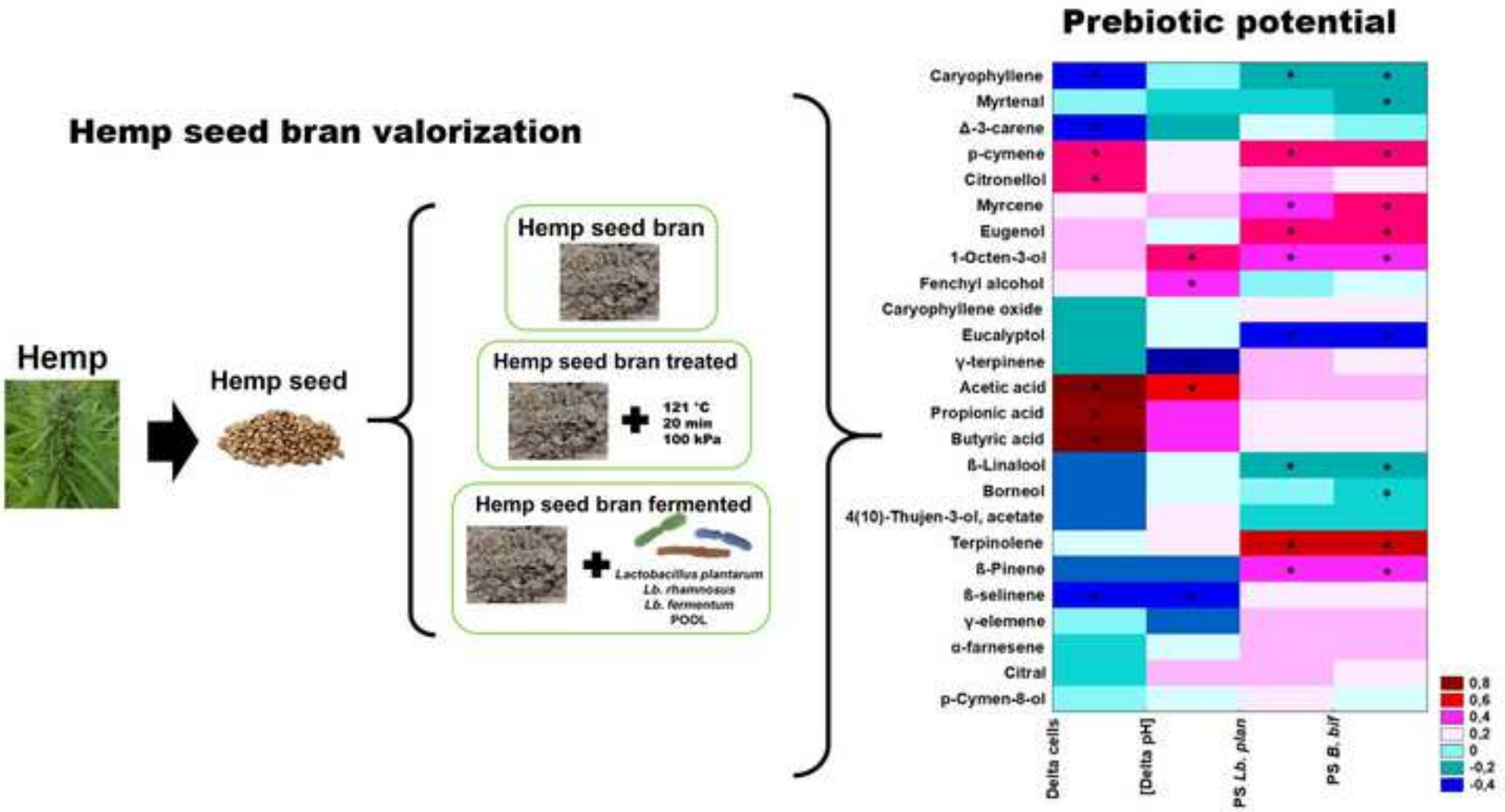

Prebiotic potential and bioactive volatiles of hemp byproduct fermented by lactobacilli

CRediT authorship contribution statement

Lorenzo Nissen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Flavia Casciano: Formal analysis, Investigation, Data curation, Investigation, Software, Writing - review & editing.

Elena Babini: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing - review & editing.

Andrea Gianotti: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of interests

☒ 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:

'Declarations of interest: none'