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Prebiotic potential and bioactive volatiles of hemp byproduct fermented by lactobacilli

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

--Manuscript Draft--

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<b>Abstract:</b>	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 <i>in vitro</i> , 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**

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

3

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#### 44 Highlights

- 45 • Hemp seed bran is an ~~unexploited-untapped~~ food source for human consumption
- 46 • ~~P~~The prebiotic ~~potentialactivity~~ is studied ~~coupling~~was studied analyzing microbial  
47 ~~growth-microbiology-~~ and bioactives ~~production~~
- 48 • ~~A bacterial pool fermented better hempseed bran than single bacterial species~~The best  
49 ~~fermentations of hemp seed bran are those made with a bacterial pool.~~

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

52

53

#### 54 Abstract

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

71

#### 72 Keywords

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

74

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

## 80 1. Introduction

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

101 dietary fibers, that bring and liberate or serve for the gut microbiota to generate different bioactive  
102 molecules such as (short chain organic acids or terpenes), could attain to this new description. The  
103 para prebiotic activity of some fibers could be improved with *in vitro* fermentation by beneficial  
104 bacteria. This strategy permits to obtain a product with Besides, it is important to consider that  
105 fermentation by lactobacilli improves the quality of the final product substantially over two aspects:  
106 firstly, the deconstruction of the fiber liberates other compounds, such the so-called postbiotics, that  
107 improves the prebiotic potential of the original product, while secondly the yield and more  
108 bioactives and to improve their bio accessibility of many different bioactives (polyphenols and  
109 terpenes) resulted augmented. These bacteria are able to ferment plant-based matrices generating  
110 and transforming metabolites. For instance, when *Lactocaseibacillus rhamnosus* ~~*Lactobacillus*~~  
111 *rhamnosus* LGG is applied in combination and different with two *Lactiplantibacillus plantarum*  
112 *subsp. plantarum* *Lb. plantarum* isolates to ferment plant-based products, improves the content of  
113 phenols and flavonoids of blueberry pomace (Yan et al., 2019). In fact, the enzymatic arsenal of  
114 lactobacilli, such as that of Strains of *Lp. plantarum* *Lb. plantarum*, *Lc. rhamnosus* *Lb. rhamnosus*,  
115 and *Limosilactobacillus fermentum* improve the content of bioactives of hemp seed products *Lb.*  
116 *fermentum* perfectly conveys them for fermentation of plant-based matrices (Nissen, Demircan,  
117 Taneyo-Saa & Gianotti, 2019; Nissen, di Carlo & Gianotti, 2020). Additionally, these species are  
118 beneficial, and some related strains own the claim of probiotics, as *Lb. rhamnosus* GG (LGG), *Lp.*  
119 *plantarum* *Lb. plantarum* K10, and *Lm. fermentum* *Lb. fermentum* ME-3 (Darby, Naudin, Luo &  
120 Jones, 2019; Kim, Huang, Park, Holzappel & Lim, 2018; Nowak, Paliwoda & Błasiak, 2019).  
121 Up today, no works were conducted exploring the functional properties of hemp seed bran (HPB)  
122 after fermentation. Due to this reason, we made ~~T~~ this work with the intention to explored  
123 characterize and valorize ~~d~~ hemp seed bran HPB (HPP) potential functionalities as a consequence of  
124 LAB (lactic acid bacteria) fermentation. by coupling its We aimed to achieve this goal by coupling  
125 prebiotic activity ~~to to~~ the release of potential ~~volatile organic~~ bioactives volatile organic  
126 compounds (VOCs) as a consequence of LAB fermentation: i), such as low organic acids, coming



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

## 137 2. Materials and methods

### 138 2.1. HPB-Hemp seed bran preparation

139 HPB, a byproduct remaining after mechanical pressing of hemp seeds and subsequent grinding and  
140 sieving, was supplied by a local company (Hemp Positive World, Cesena, Italy). Original hemp  
141 variety was Futura 75. Five grams of HPB were suspended in 30 mL of distilled water, sterilized  
142 (121 °C and 100 kPa for 20 min) (Vapor Matic 770, ASAL Srl, Milan, Italy) in independent 50 mL  
143 Falcon conical tubes (Corning Inc., NY, USA) 50 mL plastic tubes (121 °C and 100 kPa for 20  
144 min), named TBH (Treated Bran Hemp), and then used as substrate for bacterial fermentations,  
145 named FBH (Fermented Bran Hemp). Before fermentation ~~addition of the bacterial inoculum~~, the  
146 suspension was adjusted to 27 mL ~~of volume~~, in order to add later just 3 mL of bacterial inoculum.  
147 Controls used were: i) not inoculated sterile HPB/water suspension (NF = Not Fermented); ii) not  
148 sterilized nor inoculated sample (BH = Bran Hemp); iii) and a commercial hemp seed flour (HF)  
149 (Hanf & Natur, Lindlar, Germany) in a similar water suspension.

### 151 2.2. Microbial strains and culture conditions

152 All microbial strains tested belong to the microbial collection of DISTAL (Dept. of Agricultural and  
153 Food Sciences), University of Bologna (Bologna, Italy) and have been previously isolated from  
154 plant-based products and extensively studied (~~Babini, Tagliazucchi, Martini, Dei Più & Gianotti,~~  
155 ~~2017~~; Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; [Babini, et al. 2020](#); Nissen, di Carlo &  
156 Gianotti, 2020; ~~Nissen, Casciano & Gianotti, 2021~~). [Lactiplantibacillus plantarum subsp.](#)  
157 [plantarum](#) 98b, [Limosilactobacillus fermentum](#) MR13, [Lacticaseibacillus rhamnosus](#) C1112 (used  
158 for hemp bran fermentation), *Bifidobacterium bifidum* NCIMB 700795 and *Escherichia coli* ATCC  
159 25922 (used for prebiotic activity) were cultured from glycerol stocks stored at -80 °C and were  
160 propagated in selective media (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at specific  
161 conditions (Nissen, di Carlo & Gianotti, 2020).

162

### 163 2.3. Fermentations

164 The hemp seed bran samples were fermented independently by [Lc. rhamnosus](#) C1112 (C), [Lp.](#)  
165 [plantarum](#) 98b (L), [Lm. fermentum](#) MR13 (M), and by a bacterial pool (P) containing equal  
166 proportion of the aforementioned strains. Cell load of inoculated bacteria was standardized by  
167 spectrophotometric means based on plate counts and qPCR (quantitative PCR). ~~I. For each~~  
168 ~~inoculum were made by three~~ 3 mL of 7 Log<sub>10</sub> cells/mL of bacterial cells, ~~were~~ centrifuged and  
169 resuspend two times in sterile [distilled](#) water. [Each inoculum was added to 27 mL of TBH](#)  
170 ~~besuspension fore addition to TBH, whose fermentation was conducted in 30 mL. Fermentation and~~  
171 [fermentation](#) was conducted [aerobically at 37 °C up to 72 h in 50 mL Falcon conical tubes](#)  
172 [\(Corning, USA\) aerobically at 37 °C up to 72 h](#) to obtain FBH (~~Fermented HB~~) samples. Each  
173 duplicate of a time point sample was made in distinct [50 mL Falcon conical](#) tubes [\(Corning, USA\)](#).  
174 Non inoculated autoclaved hemp bran (TBH) was used as control. Two biological replicates of each  
175 sample were performed. For each inoculated sample (C, L, M, and P) sampling was performed after  
176 6, 24, 48, and 72 h as reported in Supplementary Table 1. Analyses were regarded to bacterial  
177 quantifications, pH, and VOCs (~~volatile organic compounds~~) characterization at least in duplicates.

178

#### 179 **2.4. Bacterial CFU-Culture-Dependent Counting**

180 For all bacteria 1 mL of each sample was aseptically transferred into a sterile tube ~~of~~ with 9 mL of  
181 physiological solution (0.9 g/dL NaCl) to be serially diluted (1/10) and plated in  
182 duplicates. Lactobacilli and the pool were counted on MRS agar (Oxoid, Thermo Fisher Scientific,  
183 USA) after incubation for at least 24 h at 37 °C in jars with anaerobiosis catalyst (Oxoid, Thermo  
184 Fischer Scientific, USA). *B. bifidum* NCIMB 700795 was counted on MRS agar supplemented with  
185 0.005 g/dL L-cysteine (Sigma, USA) after incubation in the same conditions of lactobacilli. *E.*  
186 *coli* ATCC 25922 was counted on BHI agar (Oxoid, Thermo Fisher Scientific, USA) at 37 °C for  
187 24 h.

188

#### 189 **2.5. pH measurement**

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

193

#### 194 **2.6. Quantification by qPCR**

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

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

205

## 206 **2.7. Prebiotic activity**

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

222

## 223 **2.8. Solid-Phase Microextraction Gas chromatography/Mass spectrometry (SPME-GC-MS)**

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

229 (Guerzoni, Vernocchi, Ndagijimana, Gianotti & Lanciotti, 2007; Di Cagno et al., 2011; Nissen,  
230 Demircan, Taneyo-Saa & Gianotti, 2019; ~~Nissen, di Carlo & Gianotti, 2020~~; Nissen, ~~Caseiano di~~  
231 Carlo & Gianotti, 2020). Briefly, before each SPME sampling, the fiber was exposed to the GC  
232 inlet for 10 min for thermal desorption at 250 °C in a blank sample. Prior analyses 6 µL of 10,000  
233 mg/mk of 2-Pentanol, 4-methyl (Merck, Darmstadt, Germany) as internal standard were injected  
234 into the vial containing 3 mL of liquid sample and let to equilibrate for 10 min at 40 °C in a water  
235 bath. The SPME fiber was exposed to each sample for 40 min, and finally the fiber was inserted  
236 into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50  
237 °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  
238 was maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230  
239 °C, respectively. Injections were carried out in split-less mode and helium (3 mL/min) was used as  
240 a carrier gas. Identification was obtained with NIST 11 MSMS library and the NIST MS Search  
241 program 2.0 (NIST, Gaithersburg, MD, USA). Acetic acid, Propionic acid, and Butyric acid were  
242 absolutely quantified in mg/kg ~~employing an internal standard (Di Cagno et al., 2011; Nissen, di~~  
243 Carlo & Gianotti, 2020) (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg), while terpenes compounds  
244 were relatively quantified from chromatogram peak areas, as a ratio peak area/total peak of different  
245 samples (Bonfrate et al. 2020) (LOD = 0.001 mg/kg) and then normalized with the mean centering  
246 method (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019; Nissen, di Carlo & Gianotti, 2020;  
247 Nissen, ~~Caseiano-Bordoni~~ & Gianotti, 2020). The samples analyzed were 3 mL of each time points  
248 case, namely 0 h, 6 h, 24 h, 48 h, and 72 h. The Samples analyzed were those collected from two  
249 technical replicas of two independent experiments.

250

## 251 **2.9. Statistical analyses**

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

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

265

### 266 3. Results

#### 267 3.1. pH values, bacterial quantifications of TBH fermentations

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

280

### 281 3.2. Volatile (low molecular weight) organic acids

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

298 In Figure 1B and Supplementary Table 5 the mean values of ~~propionic~~Propionic acid are described.  
299 ~~TFor this compound, a similar scenario to acetic acid was seen. In fact, ffrom a very low-little value~~  
300 in NF ( $0.04 \pm 0.04$  mg/kg) ~~, the production of propionic acid was raising~~raised constantly over time  
301 up to the endpoint for ~~the average of single inoculum~~C, L, and M, and up to 48 h for ~~the pool~~P.  
302 ~~Considering the mean of values of C, L, and M, Propionic acid abundance was 5.75-, 10.58-, and~~  
303 ~~14.25-folds larger at 24, 48, and 72 h. Excluding not significant early time point increase ( $P > 0.05$ ),~~  
304 ~~the increment means of the single inocula were 5.75-, 10.58-, and 14.25-folds more at 24, 48, and~~  
305 ~~72 h, respectively.~~ Otherwise, the increment values performed by ~~the pool~~P were 24.00-, 29.00-,  
306 and 22.00-folds more at 24, 48, and 72 h, respectively. Thus, ~~the pool~~P already produced higher

307 yields at 24 h, 4.17-folds more than ~~the average of single inocula~~ the mean of C, L, and M. The  
308 maximum value among the dataset was once more that of P48, scoring  $1.16 \pm 0.13$  mg/kg, 2.74-  
309 folds more than the mean of every single inoculum at that timepoint ( $P < 0.05$ ). Considering ~~the~~  
310 single ~~inocula~~ inoculum, the best performer was *M.Lb. fermentum* MR13 at 72 -h (M72), recording  
311  $0.71 \pm 0.15$  mg/kg.

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

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

### 329 3.3. Terpenes

330 Among the whole dataset of identified VOCs, we selected 37 compounds, based on their chemical  
331 class (terpenes and sesquiterpenes), normality distribution, significant difference of variance ( $P <$   
332  $0.05$ ), and ~~now~~ proved bioactivity (Figure 2). From the PCAs (Figures 4A-2A and 2B) a robust



333 plane was evidenced, based on two factors defining the 26.11% and 26.63% of total representations.

334 Coupling PCAs to K-Means clustering analysis (Figure ~~4C2C~~) it was possible to identify five

335 clusters of ~~samples-cases~~ described by significant differences ( $P < 0.05$ ) on ~~relative abundances of~~

336 30 molecules. In Figure 2A, cluster 1 (~~blue dot~~) was positioned on quadrant III of PCA's plane

337 oriented distant to the left side and grouped just NF samples. This cluster was described by 30

338 compounds but just eight had relative higher ( ~~$P < 0.05$~~ ) abundances ( ~~$P < 0.05$~~ ) than FBH samples,

339 such as  ~~$\beta$ -pinene~~ Pinene,  $\gamma$ -Elemene, cis- $\beta$ -Farnesene, Aromadendrene, 9-

340 ~~methyldecalin~~ Methyldecalin,  $\alpha$ -Farnesene, Geraniol, and Myrtenal. Thereof, all other ~~samples-cases~~

341 ( $n = 32$ ) were ~~relative to the~~ FBH ~~ones-samples and were~~ distributed in four specific clusters.

342 Cluster 2 (~~fuchsia dot~~) included ~~early time point~~ ~~the samplecases at the early time points plus~~

343 ~~two relatives to 24 h time point~~ and other two (L24 and L24\_2) ~~and was mainly fitted in quadrant IV~~

344 ~~of PCA's plane~~. This cluster was described by 17 variables, but just three ~~had abundancies~~

345 ~~relatively higher~~ were more abundant than ~~those found in~~ other clusters ( $P < 0.05$ ), *i.e.* butylated

346 Butylated hydroxytoluene, trans-Pinocarveol, and *p*-Vinylguaiacol. Cluster 3 included ~~just MR13~~

347 ~~and LB325 fermented samples~~ FBH samples fermented by M and L at intermediate and end time

348 points. ~~In particular, M13 at 24, 48, and 72 h time points, while LB325 at 48 and 72 h time points.~~ It

349 was described by 24 compounds, but none ~~had abundancies~~ was significantly higher than ~~those of~~

350 other clusters ( $P > 0.05$ ). Cluster 4 included all ~~FBH samples~~ the pool (P) fermented by P samples

351 except that at the early time point ~~and it was set in quadrant I of PCA's plane oriented to the top~~. It

352 was described by 24 compounds, ~~whose and~~ eight ~~had abundancies~~ were significantly ~~higher more~~

353 ~~abundant~~ ( $P < 0.05$ ) than those of other clusters, *i.e.* Caryophyllene oxide, 1-(R)- $\alpha$ -Pinene,

354 Eudesma-4(14),11-diene, *p*-Cymene, Myrcene,  $\Delta$ -3-Carene, 1-Octen-3-ol, and Citronellol. Cluster 5

355 contained ~~all the cases related to intermediate and end time point~~ FBH samples fermented by C112

356 ~~fermented samples relatives to intermediate and end time points and was positioned in quadrant IV~~

357 ~~of PCA's plane~~. It was described by 23 terpenes ~~in varying abundancies, among which~~ and in

358 ~~particular~~ that of 4-Trimethylsilyl-9,9-dimethyl-9-silafluorene, 4(10)-Thujen-3-ol, acetate, and

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

#### 364 3.4. Targeted MANOVA: fermentation dynamics and strain performances

365 MANOVA ( $P < 0.01$ ) was performed on the dataset of the 37 normally distributed variables  
366 with two categorical predictors: i) bacterial inoculum and ii) on the time of  
367 fermentation to perform MANOVA ( $P < 0.01$ ) (Figure 3A and B Tables 1 and 2), in order to address  
368 specifically the production of terpenes. Considering the different fermenting agent bacterial  
369 inoculum (Figure 3A Table 1), 20 variables had significant differences ( $P < 0.01$ ) and it emerged  
370 that not fermented samples NF (Control) samples were the sole account described by Geraniol  
371 (100%), and for more than the 76.0% of total 9-Methyldecalin (76.0%), and 72.1% of  $\beta$ -Selinene  
372 (72.1%) abundances. The quantities of the remaining 17 VOCs were all significantly augmented  
373 with fermentation. In particular, some compounds were produced in higher proportion by a given  
374 inoculum in respect to the others, and differently by the inoculum. For example, C1112 was  
375 responsible for 48.9% of total production of  $\alpha$ -Caryophyllene, 40.6% of Borneol, and 43.2% of  
376 Eucalyptol. Fermentation by LB325 led to the production of 52.9% of total  $\beta$ -Linalool and 37.7% of  
377 total *p*-Cymen-8-ol. Fermentation conducted by MR13 was distinguished by 49.9% of total  
378 abundance of *p*-Cymene and by 50.5% of total Myrtenal. The pool P was responsible for the 59.9%  
379 of the total yield in 1-(R)- $\alpha$ -pinene Pinene, for the 63.9% of total  $\gamma$ -Elemene, the 57.6% of total cis-  
380  $\beta$ -Farnesene, the 51.8% of total Myrcene, the 44.8% of total  $\Delta$ -3-carene Carene, the 47.8% of total  
381 Fenchyl alcohol, the 45.8% of total 4(10)-Thujen-3-ol, acetate, and the 45.8% of total Eugenol. So  
382 far, TBH fermented by the pool showed to be the inoculum that accounted for higher production of  
383 more compounds than the single strains. In fact, the pool P produced had higher amount of 9  
384 terpenes in comparison to the 3 of C1112, and the 2 of both LB325 and MR13.

385 ~~Instead, considering the~~ MANOVA categorized for the time points of fermentation ~~showed that,~~ 20  
386 ~~compounds VOCs~~ had significant differences ~~among the independent variables~~ ( $P < 0.01$ ) (Figure  
387 ~~Table 23D~~). Seven terpenes were intrinsic features of HPB and were not subject to significant  
388 ~~increases through with~~ fermentation, ~~in particular:~~  $\beta$ -Pinene, ~~and Geraniol, and  $\alpha$ -Farnesene was~~  
389 ~~were almost not detected after fermentation~~ an exclusive signature of NF samples. ~~The other~~  
390 ~~compounds were:~~ Aromadendrene, accounting for the 66.4% of total abundance, 9 Methyldecalin,  
391 ~~accounting for the 76.0%,  $\beta$  Solinene for the 72.1%, and  $\alpha$  Farnesene for the 94.6%.~~ For any ~~other~~  
392 ~~compound,~~ the abundance was in higher proportion at the late time points. ~~In fact~~ ~~In brief,~~ after 24  
393 ~~h just the 33.7% of total Eucalyptol and the 62.6% of total p-Vinylguaiacol were produced. Instead,~~  
394 ~~after at~~ 48 h the ~~VOCs discriminated were:~~ 70.9% of total Eudesma-4(14), 11-diene, (70.9%), as  
395 ~~well as the 50.9% of total p-Cymene (50.9%), the 56.6% of total Terpinolene (56.6%), the 40.6% of~~  
396 ~~total 1-Octen-3-ol (40.6%), and the 42.1% of total 2-Decen-1-ol, (E) (42.1%) were produced.~~ Lastly,  
397 at the end point major proportions on total yields of  $\gamma$ -Elemene (84.4%), of Citronellol (38.6%), and  
398 of Myrtenal (54.4%) were achieved.

399

### 400 3.5. Prebiotic score

401 ~~The prebiotic scores were calculated from the equation proposed by Huebner, Wehling & Hutkins,~~  
402 ~~(2007) and revised by Fissore, Santo Domingo, Gerschenson & Giannuzzi, (2015), which considers~~  
403 ~~the effect of a fiber in comparison to glucose towards the growth of a beneficial bacteria in respect~~  
404 ~~to the growth of pathogenic *E. coli*.~~ The highest score for prebiotic activity (Table 43) versus *Lp.*  
405 ~~plantarum *Lb. plantarum* 98b~~ was achieved by P48, that was the sole sample scoring significantly  
406 higher than FOS ( $P < 0.05$ ). ~~In fact~~ ~~in particular,~~ ~~TBH fermented by P48 was significantly stronger~~  
407 ~~than FOS in the containment of *E. coli* ATCC 25922 even if the growth of *Lb. plantarum* 98b on 1~~  
408 ~~g/dL of TBH fermented by P48 was slightly lower than that of FOS, the inhibition of the former on~~  
409 ~~*E. coli* ATCC 25922 was significantly stronger~~ ( $P < 0.05$ ) (Supplementary Table 6). ~~Besides, in~~  
410 ~~comparison to FH, TBH fermented by P48~~ P48 reached a prebiotic score ~~on~~ had a prebiotic score

411 1.8-folds higher versus *Lp. plantarum* 98b ~~*Lb. plantarum* 98b~~ 1.8 folds higher than FH that had the  
412 lowest value. The ~~prebiotic score raised in respect to~~ score raised in respect to the intensity of HPB  
413 treatment, ~~in details BH had the lowest value and TBH the highest~~ from the lowest of BH to the top  
414 of fermented TBH. Among the fermented samples the runner up was C48, with a score slightly  
415 lower than FOS, but significantly higher than similar samples (L48 and M48). Considering the  
416 prebiotic activity towards *B. bifidum* NCIMB 700795 (Table 43), ~~a similar trend was evidenced.~~  
417 The best performing sample was C48, higher than FOS and P48, but with no significant difference  
418 ( $P > 0.05$ ). ~~Even in this context, Similarly to the previous prebiotic target, FOS made foster more~~ *B.*  
419 *bifidum* NCIMB 700795 ~~to grow more~~ than the best TBH fermented sample (C48), but this latter  
420 was stronger in ~~the containment of~~ *E. coli* ATCC 25922 inhibition ( $P < 0.05$ ) (Supplementary Table  
421 6). Besides, TBH fermented by C48 hit the top prebiotic score versus *B. bifidum* NCIMB 700795  
422 and was ~~Significantly~~ different from any other samples ( $P < 0.05$ ) ~~ees were seen in respect to all~~  
423 other samples, and in particular the prebiotic activity of C48 was 1.7 folds more effective than that  
424 of FH. Thus, both C48 and then P48 scored higher values than other fermented or not fermented  
425 samples ( $P < 0.05$ ). In brief, among the strains tested after 48 h of fermentation ~~of the pool~~ TBH, the  
426 pool demonstrated to produce a substrate ~~that had the top~~ with the best prebiotic activity versus  
427 ~~lactobacilli~~ *Lp. plantarum*, while C112 hit the top with the best versus *B. bifidum* bifidobacteria.

### 429 3.6. Spearman rank correlations

430 We used Spearman rank analysis to evidence ~~d~~ correlations between variables related to ecological  
431 features (bacterial growth, pH decrease, and prebiotic activity) and those related to abundances of  
432 VOCs ~~considered~~, on independent variables ( $n = 32$ ) (Figure 43). ~~Considering the bacterial growth,~~  
433 The variable “delta cells” ~~indicates the was obtained from the~~ difference ~~of in~~  $\text{Log}_{10}$  cells/mL at the  
434 ~~endpoint and~~ between the beginning and end of fermentation. Significant correlations ( $P < 0.05$ )  
435 ~~indicated~~ evidenced that during fermentation of TBH samples the ~~the more~~ growth of bacterial grew  
436 in fermentation the more was positively correlated with quantity of SCFAs, and minorly  $p$ -

437 Cymene, and Citronellol ~~was found in fermented TBH samples~~. In contrast three terpenes, *i.e.*  
438 Caryophyllene, Δ-3-Carene, and β-Selinene, were inversely ~~proportional-correlated~~ to bacterial  
439 growth. ~~It is likely that their accumulation in the substrate resulted in a constraint for lactobacilli.~~  
440 ~~As a matter of fact, Considering that from previous MANOVA ( $P < 0.05$ ) resulted that the longer~~  
441 ~~was the fermentation time the larger the quantity of these three VOCs from MANOVA was found~~  
442 ~~that almost 50% of total yield of these VOCs was fostered by fermentation, it is likely that their~~  
443 ~~accumulation in the substrate resulted in a constraint for lactobacilli as the fermentation was~~  
444 ~~prolonged over time when bacterial load was richer.~~  
445 ~~Acetic acid abundance was even significantly proportional to acidification, as well as that of 1-~~  
446 ~~Octen-3-ol and Fenchyl alcohol.~~ Considering the prebiotic activity, it is interest to stress out that  
447 correlation trend was similar for both probiotics. A group of ~~terpenes-VOCs~~ including *p*-Cymene,  
448 Myrcene, Eugenol, 1-Octen-3-ol, Terpinolene, and β-Pinene resulted significantly associated to  
449 prebiotic activity ( $P < 0.05$ ), while Caryophyllene, Eucalyptol, and β-~~linalool-Linalool~~ were  
450 ~~negative~~inversely ~~proportional-correlated~~ ( $P < 0.05$ ). This issue could mean that ~~just the former list~~  
451 ~~of VOCs related to prebiotic activity has-generate a selective bioactivity versus certain~~  
452 ~~baeriaeffects, e.g. inhibiting-inhibition of enteropathogenic *E. coli* enteropathogens and capacity~~  
453 ~~to fostering probiotics *B. bifidum* or *lactobacilli* *Lp. plantarum*, instead the latter list of VOCs had a~~  
454 ~~broader spectrum of antimicrobial activity.~~

455

#### 456 4. Discussion

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

463 Garden-Robinson & Hall, 2017). Kimchi fermentation by indigenous LAB, ~~including *Laetobacillus*~~  
464 ~~spp.~~, can acidify the substrate up to pH 3.5 after 24 h (Joon-Yeon & Kunz, 2009). In our work, from  
465 24 h to the ~~endtime~~ point the pH ~~remained-was~~ stable with a mean ~~value~~ of  $4.41 \pm 0.15$ , ~~besides-and~~  
466 ~~in-this-period~~ bacterial cells kept growing up to ~~an-endpoint~~ a mean of  $12.23 \pm 0.24 \text{ Log}_{10} \text{ cells/mL}$ .  
467 Therefore, HPB positively ~~reflects the essential~~ ~~showed characteristic of~~ ~~ato be a~~ substrate ~~for which~~  
468 ~~fosters~~ probiotic growth ~~including-and owns~~ a pH buffering capacity (Nissen, di Carlo & Gianotti,  
469 2020).

470 The prebiotic activity ~~scored-recorded~~ by fermented TBH was surprisingly effective even due to a  
471 stronger containment of the growth of *E. coli* ATCC 25922, in comparison to other samples and the  
472 FOS. ~~Hemp female inflorescences and hemp seeds bring many terpenes with renowned~~  
473 ~~antimicrobial activity~~ (Nissen et al., 2010; Pellati, Brighenti, Sperlea, Marchetti, Bertelli &  
474 ~~Benvenuti et al., 2018; Leghissa, Hildenbrand & Schug, 2018), that alone or in synergism show to~~  
475 ~~be capable to inhibit opportunistic and food borne pathogens~~ (Nuutinen, 2018; Nafis et al., 2019).  
476 ~~The antimicrobial activity of hemp seed is reckoned to be triggered by the synergistic effects of~~  
477 ~~different terpenes present in hemp seed oil~~ (Nafis et al., 2019; Nissen et al., 2010). On the  
478 contrary, other plant-based materials able to foster probiotics do not have a prebiotic activity  
479 because cannot tackle the growth of enteropathogens (Vieira, Bedani, Albuquerque, Biscola &  
480 Saad, 2017). A fundamental criterium to classify a food ingredient as a prebiotic is the scientific  
481 ability to foster the growth and support the activity of beneficial intestinal bacteria (Gibson et al.  
482 2017). In this view the assay of prebiotic activity adopted reflects the ability of a prebiotic to  
483 ~~jointly mutually~~ foster the growth of probiotics and limit that of enteropathogens in comparison to  
484 glucose (Fissore, Santo Domingo, Gerschenson & Giannuzzi, 2015; Huebner, Wehling, Parkhurst  
485 & Hutkins, 2008). ~~Another element that supports the~~ ~~The s~~ strong prebiotic activity of FBH that we  
486 ~~have~~ observed could be ~~partly due to the~~ ~~recorded~~ higher ~~quantity~~ levels of ~~aeetic~~ Acetic,  
487 ~~propionic~~ Propionic, and ~~butyric~~ Butyric acids ~~generated s,~~ ~~particularly by fermentation with in~~ P48  
488 and in C72. Lactobacilli are able to liberate and produce low organic acids during fibers

489 degradation, thus improving the original content in the fermented product (Massa et al., 2020). The  
490 quality and quantity of these organic acids depend on the type of fiber used (Gill, van Zelm, Muir &  
491 Gibson 2018).  
492 ~~In fact,~~ the beneficial effects of low organic acids are renowned ~~and are multi-targets, not solely~~  
493 ~~directed to the host epithelial mucosa, and to the blood stream, but even to the microbiota, as a~~  
494 ~~selective substrate (Goverse et al., 2017). A~~ abundant production of these compounds is linked to  
495 well-being (Goverse et al., 2017) and their nutritional supplementation ~~are is~~ suggested for the  
496 treatment of ~~in~~ different intestinal diseases (Gill, van Zelm, Muir & Gibson 2018).  
497 From Spearman Rank correlation, the abundance of *p*-Cymene, Myrcene, Eugenol, 1-Octen-3-ol,  
498 Terpinolene, and  $\beta$ -Pinene was linked to prebiotic activity, and all these VOCs, except the latter,  
499 were increased with fermentation of TBH. These results are in line with recent literature, where it is  
500 reported that bacterial fermentation is able to improve the original terpenes content of cheese (Alves  
501 Bezerra et al., 2017), mango peel (Jin et al., 2018), okara from soybean (Gupta, Lee & Chen, 2018),  
502 hemp seed drinks (Nissen, di Carlo & Gianotti, 2020), hemp seed enriched doughs (Nissen,  
503 Bordoni, Gianotti, 2020), and blueberry pomace (Cheng et al., 2020). The antimicrobial properties  
504 and the applications in foods of terpenes such as *p*-Cymene (Marchese et al., 2017), Terpinolene  
505 (Fiorini et al., 2019; Karas et al., 2020), Myrcene (Mitropoulou et al., 2107), and Eugenol (Talón,  
506 Vargas, Chiralt & González-Martínez, 2019) is proved. Additionally, these VOCs are promising  
507 health related compounds due to the strong anti-oxidant and anti-inflammatory capacity, as good  
508 radicals' scavengers (De Oliveira et al., 2015; Boulebd, 2021; Yi, Sun, Bao, Ma & Sun, 2019; da  
509 Silva et al., 2018). Myrcene that was plenty in FBH samples. In fact, this monoterpene rules almost  
510 a third of Futura 75 hemp seed essential oil content (Nissen et al., 2010), and besides has  
511 antioxidant and anti-inflammatory capacities (Mitropoulou et al., 2107; Yi, Sun, Bao, Ma & Sun,  
512 2019). Then we found high abundance of Terpinolene, that is a monoterpene and a structural isomer  
513 of (+) Limonene that is found largely in hemp inflorescence up to 9.7% of total weight, and is  
514 currently used in the food industry (Fiorini, Molle, Nabissi, Santini, Benelli & Maggi, 2019). It has

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

524 ~~Thus, according to the new definition of prebiotics, FBH had an higher prebiotic score than FOS~~  
525 ~~because its effect was jointly generated by other beneficial compounds and the polysaccharides. In~~  
526 ~~fact, considering metabolomics, multivariate analysis defined that HPB samples prior fermentation~~  
527 ~~were described by 28 different terpenes, whose two were exclusively found at this stage, such as  $\beta$ -~~  
528 ~~Pinene and Geraniol. The other compounds were all positively subjected to the effect of~~  
529 ~~fermentation process, which have surged their release. Consequently, in our study the more the~~  
530 ~~terpenes-bioactive VOCs (low organic acids and terpenes) were abundant and the more the prebiotic~~  
531 ~~activity was effective. microbial fermentation is able to improve the original terpenes content of~~  
532 ~~cheese (Alves Bezerra et al., 2017), mango peel (Jin et al., 2018), okara from soybean (Gupta, Lee~~  
533 ~~& Chen, 2018), hemp seed drinks, and hemp seed enriched doughs (Nissen, di Carlo, Gianotti,~~  
534 ~~2020; Nissen, Bordon, Gianotti, 2020), and blueberry pomace (Cheng et al., 2020). Actually, from~~  
535 ~~Spearman-Rank correlation, the abundance of *p*-Cymene, Myrcene, Eugenol, 1-Octen-3-ol,~~  
536 ~~Terpinolene, and  $\beta$  Pinene was linked to prebiotic activity, and all these VOCs, except the latter,~~  
537 ~~were increased throughout fermentation of TBH.~~

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



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

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

551 Among the most effective terpenes that we have detected and described there were five that recently  
552 attracted scientist for their biological activities, namely Myrcene, Terpinolene, Eugenol, p-Cymene,  
553 and l-Octen-3-ol. The first is Myrcene that was plenty in FBH samples. In fact, this monoterpene  
554 rules almost a third of Futura 75 hemp seed essential oil content (Nissen et al., 2010), and besides  
555 has antioxidant and anti-inflammatory capacities (Mitropoulou et al., 2007; Yi, Sun, Bao, Ma &  
556 Sun, 2019). Then we found high abundance of Terpinolene, that is a monoterpene and a structural  
557 isomer of (+) Limonene that is found largely in hemp inflorescence up to 9.7% of total weight, and  
558 is currently used in the food industry (Fiorini, Molle, Nabissi, Santini, Benelli & Maggi, 2019). It  
559 has been reported to have antioxidant capacities as a good radicals' scavengers (Boulebd, 2021),  
560 and to act as antimicrobial (Karas, Wong, Paulin, Mazeh, Hussein, Li & Velkov, 2020). Eugenol is  
561 a natural phenolic compound found abundantly in cinnamon and in clove essential oils and is the  
562 main responsible for clove aroma (Talón, Vargas, Chiralt & González-Martínez, 2019). Eugenol is  
563 indicated for several therapeutic effects, because is a good radicals' scavengers with antibacterial  
564 effect (da Silva, Monte, de Lemos, do Nascimento, Costa, de Paiva, 2018). p-Cymene is a  
565 monoterpene found in more than 100 plant species able to counteract different food borne

566 pathogens (Marchese et al., 2017). It shows numerous biological activities, increasing the activity of  
567 antioxidant enzymes, contributing to reduce oxidative stress (De Oliveira et al., 2015).  
568 1-Octen-3-ol has been described in different plants and fungi and is a short-chain oxylipin,  
569 oxygenated derivative of linoleic acids, able to protect plants in response to external challenges  
570 (Zhang et al., 2021) and successfully tested *in vitro* against food-borne pathogens (Xiong, Li, Li,  
571 Chen, Chen, Huang, 2017).  
572 The bioactives that we have described use to act in synergism, resulting to exalt the beneficial effect  
573 for the host (Nafis et al., 2019; Nissen et al., 2010; Russo, 2011); for example, it has been proposed  
574 that phytocannabinoids and terpenoids interact together to deliver joint effect in the treatment of  
575 inflammation and microbial pathogenesis (Nuutinen, 2018).  
576 In this way a human colonic model may represent the proper *in vitro* approach for a deep  
577 knowledge of potential functionalities of HPB (Nissen, Casciano, Chiarello, Di Nunzio, Bordoni &  
578 Gianotti, 2021). Throughout the aid of such tools it will be possible to landscape the complexity of  
579 the shift of the microbial populations, as restraint of enteropathogens and promotion of probiotics,  
580 and to reveal in detail the metabolic fate of bioactive VOCs.

581

## 582 5. Conclusions

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

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

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

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

612

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### 616 **Credit authorship contribution statement**

617 **Lorenzo Nissen:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology,  
618 Software, Supervision, Visualization, Writing - original draft, Writing - review & editing.  
619 **Flavia Casciano:** Formal analysis, Investigation, Writing - review & editing.  
620 **Elena Babini:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing  
621 - review & editing.  
622 **Andrea Gianotti:** Conceptualization, Data curation, Funding acquisition, Methodology, Project  
623 administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing -  
624 review & editing.

625

#### 626 **Declaration of Competing Interest**

627 The authors declare that they have no known competing financial interests or personal relationships  
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629

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899  
900 **Figure captions**

901 Figure 1. A) Acetic acid, B) Propionic acid, and C) Butyric acid quantification by SPME GC-MS of  
902 not fermented (NF) and fermented hemp bran (FBH), expressed in mg/kg. Plots are indicating  
903 results from two different replications and two independent experiments. Boxes indicate means  
904 values; rectangles indicate means values  $\pm$  S.D.; whiskers indicate means values  $\pm$  1.96\*S.D.

905 NF = not fermented TBH; C6, C24, C48, and C72 = TBH fermented by *Lactocaseibacillus*  
906 *rhamnosus* C1112 after 6 h, 24 h, 48 h, and 72 h; L6, L24, L48, and L72 = TBH fermented by  
907 *Lactiplantibacillus plantarum subsp. plantarum* LB325 after 6 h, 24 h, 48 h, and 72 h; M6, M24,  
908 M48, and M72 = TBH fermented by *Limosilactobacillus fermentum* MR13 after 6 h, 24 h, 48 h, and  
909 72 h; P6, P24, P48, and P72 = TBH fermented by the pool after 6 h, 24 h, 48 h, and 72 h. Means  
910 with different letters are significantly different at  $P < 0.05$  by Student T-test.

911  
912 Figure 2. Multivariate analysis on 37 VOCs terpenes quantified by SPME GC-MS of not fermented  
913 (NF) and fermented hemp bran (FBH) samples. (A) PCA of cases; (B) PCA of variables; (C) K-  
914 Means clusterization based on dependent variables. Cluster 1 = blue plot; Cluster 2 = fuchsia plot;  
915 Cluster 3 = green plot; Cluster 4 = black plot; Cluster 5 = yellow plot. \*1,4,7, Cycloundecatriene,  
916 1,5,9,9-tetramethyl-, Z,Z,Z-; \*\*4-Trimethylsilyl-9,9-dimethyl-9-silafluorene; \*\*\*Phenol, 2,4-bis(1,1-  
917 dimethylethyl)-. Codes of samples: X0 = not fermented samples; C6, C24, C48, and C72 = TBH  
918 fermented by *Lactocaseibacillus rhamnosus* C1112 after 6 h, 24 h, 48 h, and 72 h; L6, L24, L48,  
919 and L72 = TBH fermented by *Lactiplantibacillus plantarum subsp. plantarum* LB325 after 6 h, 24  
920 h, 48 h, and 72 h; M6, M24, M48, and M72 = TBH fermented by *Limosilactobacillus fermentum*

921 MR13 after 6 h, 24 h, 48 h, and 72 h; P6, P24, P48, and P72 = TBH fermented by the pool after 6 h,  
922 24 h, 48 h, and 72 h.

923

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

927

928 Figure 43. Two-way joining heatmap of double matrix Spearman rank correlations on VOCs  
929 terpenes of 32 independent variables from treated hemp bran (TBH), including not fermented TBH  
930 and TBH fermented (FBH) for 6 h, 24 h, 48 h, and 72h at 37 °C by *Lactocaseibacillus rhamnosus*  
931 C1112, *Lactiplantibacillus plantarum subsp. plantarum* LB325, *Limosilactobacillus fermentum*  
932 MR13, and by the pool of these three strains. \*  $P < 0.05$ . X Axis labels: Delta cells = Log cells/ml  
933 increase; [Delta pH] = Acidification of substrate; PS *Lb. plan* = Prebiotic Score on  
934 *Lactiplantibacillus plantarum subsp. plantarum* 98b; PS *B. bif* = Prebiotic Score on  
935 *Bifidobacterium bifidum* NCIMB 700795.

936



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

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

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

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

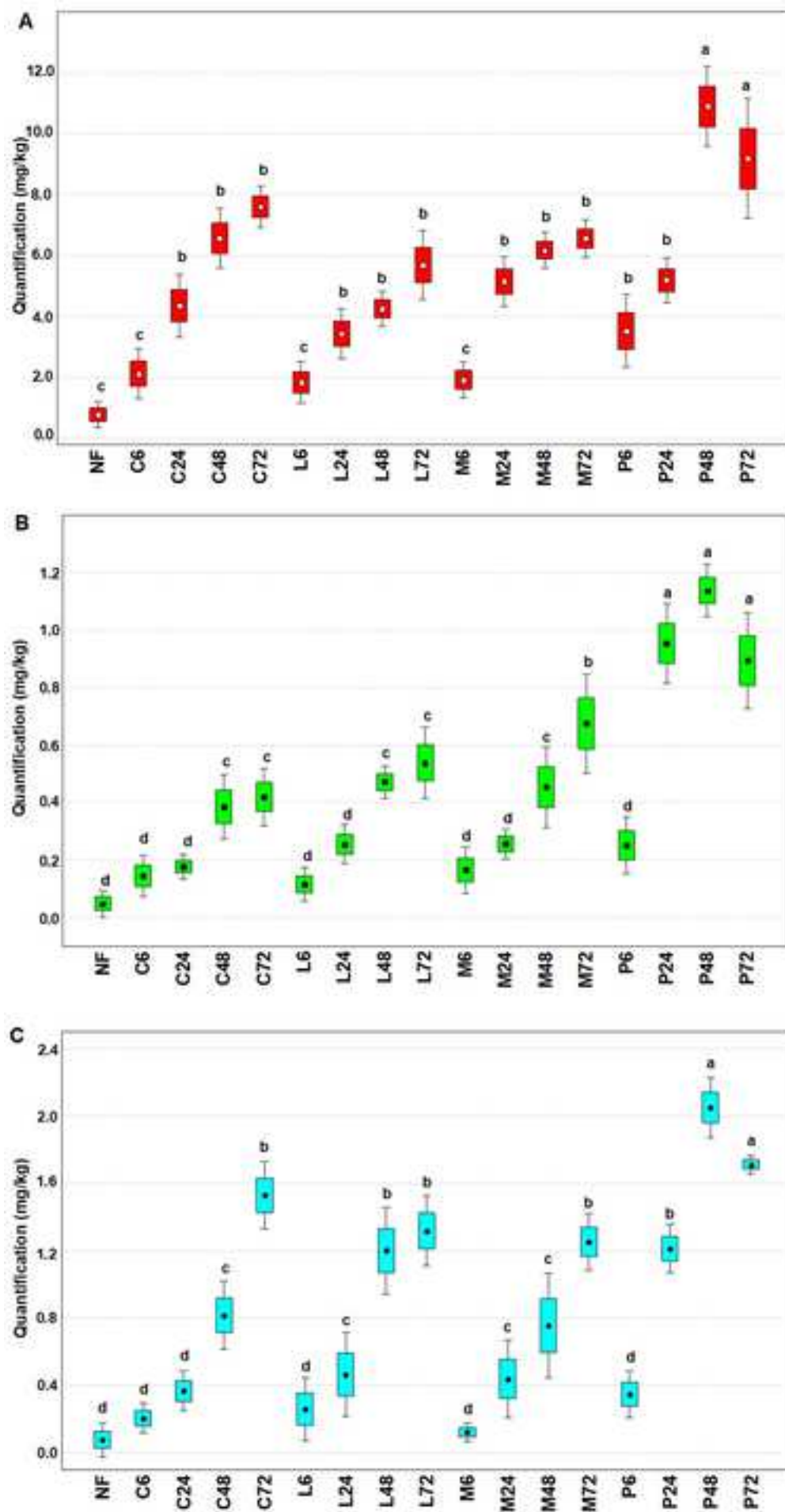
VOC	0 h*	6 h	24 h	48 h	72 h	P value
$\beta$ -Pinene	100.00 <sup>a</sup>	0.00 <sup>b</sup>	0.00 <sup>b</sup>	0.00 <sup>b</sup>	0.00 <sup>b</sup>	<0.00001
Eudesma-4(14), 11-diene	2.42 <sup>c</sup>	18.42 <sup>b</sup>	0.00 <sup>c</sup>	70.92 <sup>a</sup>	8.24 <sup>b</sup>	0.01642
p-Cymene	20.47 <sup>b</sup>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

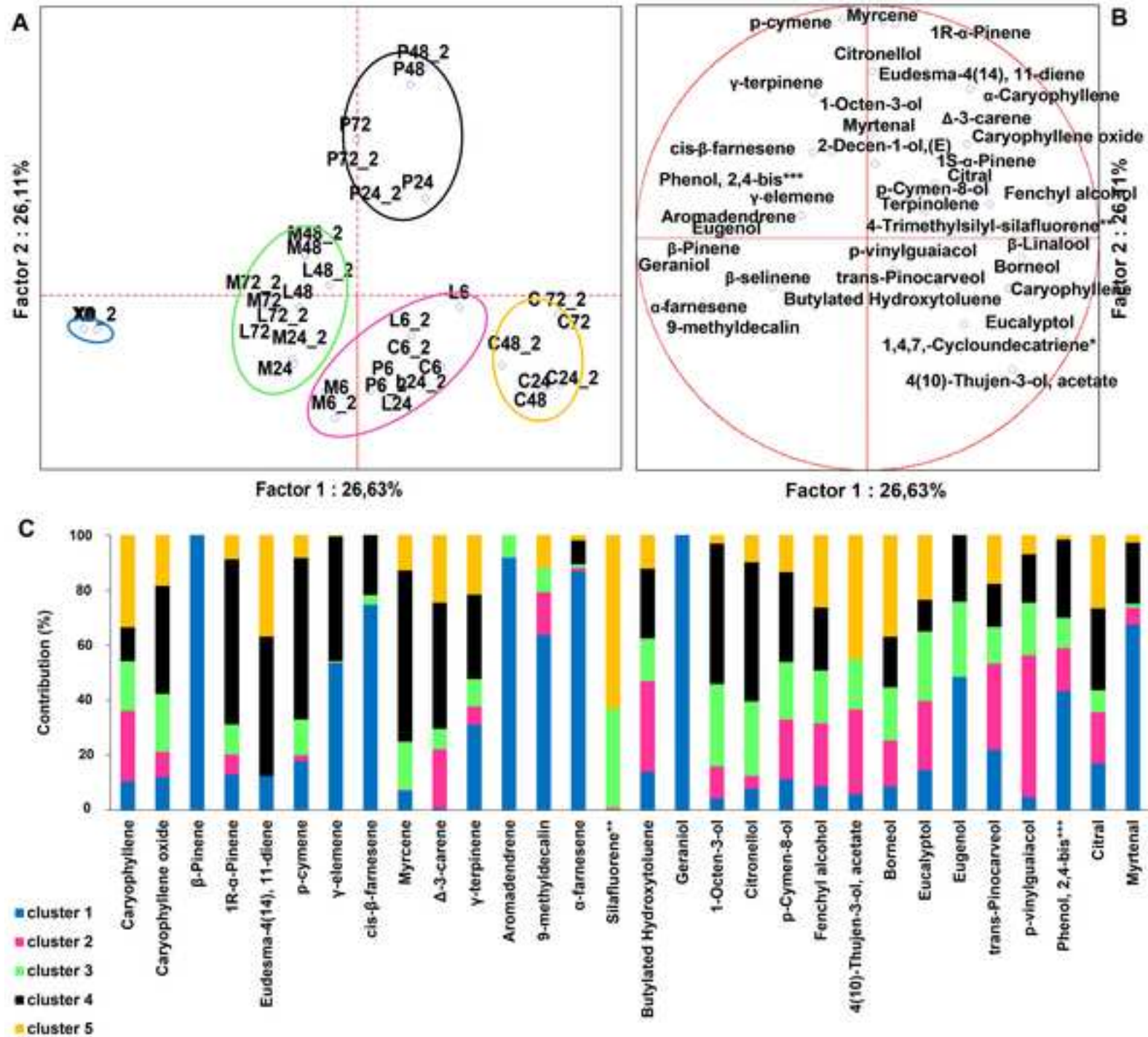
<sup>abc</sup>Different letters indicate statistical significance by Tukey's HSD post-hoc test ( $P < 0.05$ ); \*hours of fermentation.

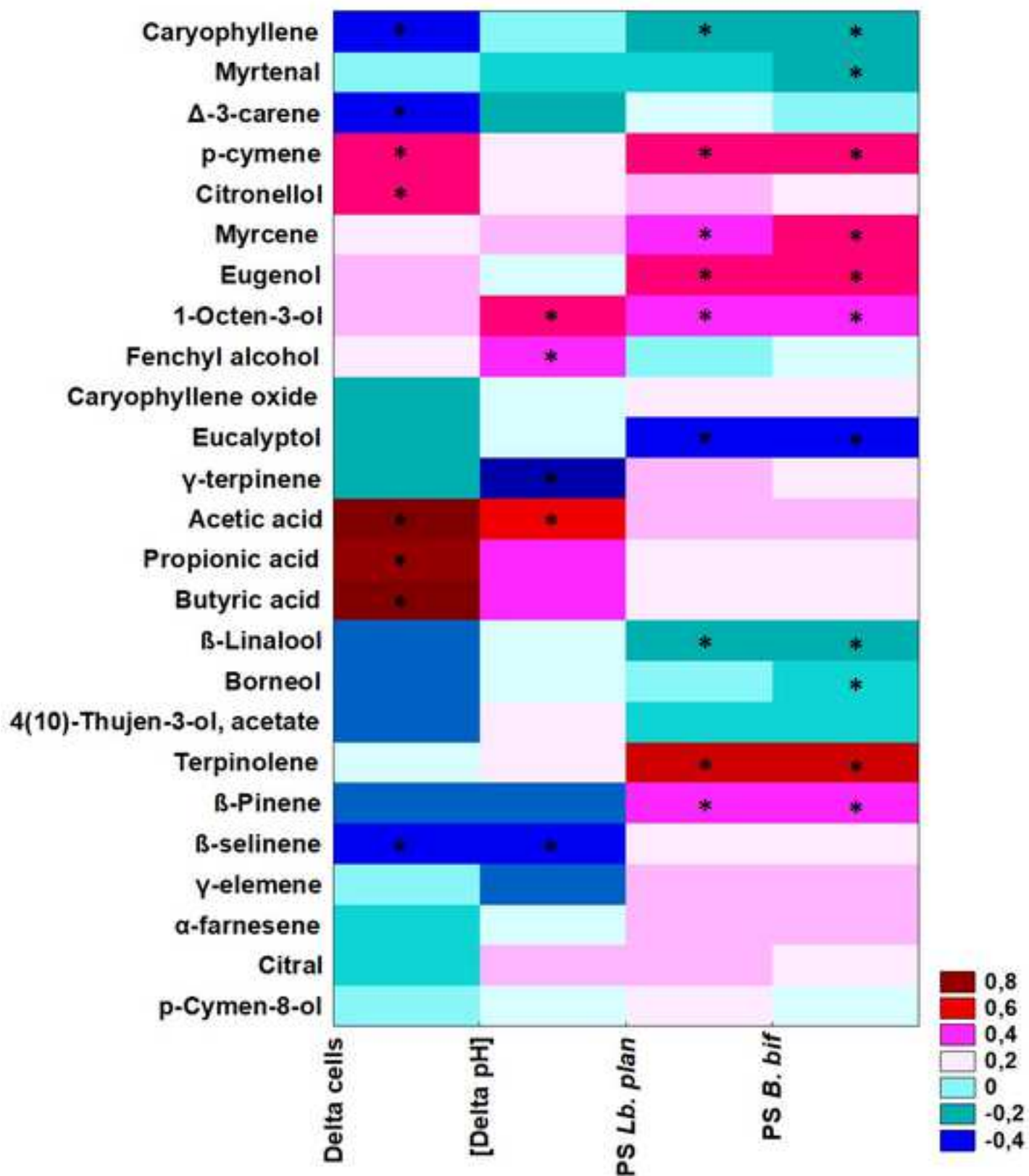
Table 1. Prebiotic activity scores obtained from cell density ( $\text{Log}_{10}$  cells/ml) of bacterial cultures grown with 1 g/dL of differently treated hemp seed bran as carbohydrate sources.

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

Values are means of three different replications and two independent experiments ± standard deviation. <sup>a,b,c,d</sup> = 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.

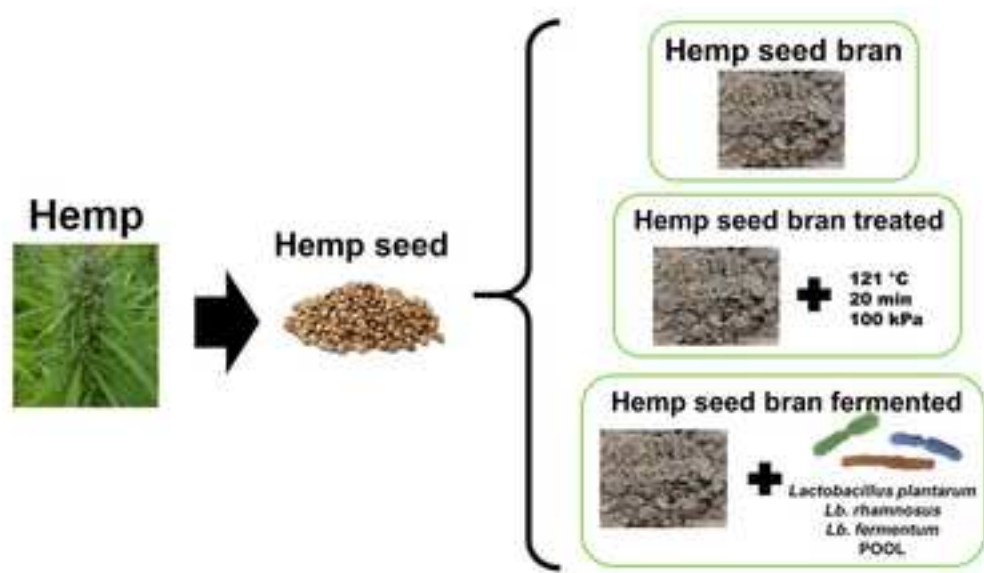




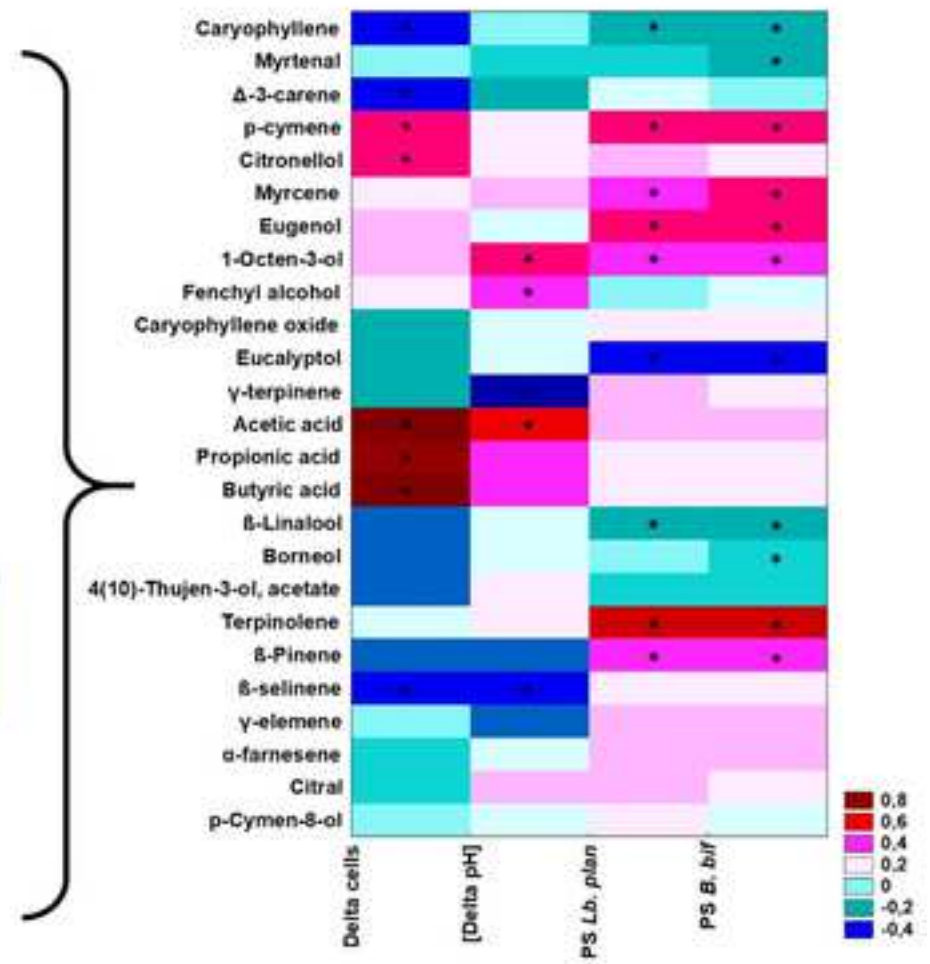




### Hemp seed bran valorization



### Prebiotic potential



## 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'