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# UCC

**University College Cork, Ireland**  
Coláiste na hOllscoile Corcaigh

1 **Pediocin PA-1 production by *Pediococcus pentosaceus* ET34 using non-detoxified**  
2 **hemicellulose hydrolysate obtained from hydrothermal pretreatment of sugarcane**  
3 **bagasse**

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## 45 **Abstract**

46

47 *Listeria monocytogenes* is one of the foodborne pathogens of most concern for food  
48 safety. To limit its presence in foods, bacteriocins have been proposed as natural bio-  
49 preservatives. Herein, a bacteriocin was produced on hemicellulose hydrolysate of  
50 sugarcane bagasse by *Pediococcus pentosaceus* ET34, whose genome sequencing  
51 revealed an operon with 100% similarity to that of pediocin PA-1. ET34 grown on  
52 hydrolysate-containing medium led to an increase in the expression of PA-1 genes and a  
53 non-optimized purification step sequence resulted in a yield of 0.8 mg·L<sup>-1</sup> of pure  
54 pediocin (purity > 95%). Culture conditions were optimized according to a central  
55 composite design using temperature and hydrolysate % as independent variables and  
56 validated in 3-L Erlenmeyers. Finally, a process for scaled-up implementation by sugar-  
57 ethanol industry was proposed, considering green chemistry and biorefinery concepts.

58 **This work stands up as an approach addressing a future proper sugarcane bagasse**  
59 **valorisation for pediocin production.**

60

61

62 **Keywords:** sugarcane bagasse; *Pediococcus pentosaceus*; bacteriocin; hemicellulose  
63 hydrolysate.

64

## 65 **1. Introduction**

66 Bacterial infections are again becoming an imminent threat to public  
67 health worldwide, jeopardizing the extraordinary clinical advances achieved since  
68 antibiotic discovery (Tacconelli and Pezzani, 2019). Due to the rise in antibiotic  
69 multi-resistance among an increasing number of pathogenic microorganisms, the  
70 search for new antimicrobial compounds will become one of the most important

71 scientific issues of the coming years (Mantravadi et al., 2019). In addition to the  
72 related medical challenges, the increasing number of disease outbreaks caused by  
73 foodborne pathogenic microorganisms, e.g *Listeria monocytogenes* has raised  
74 concerns among world public health organizations (Bucur et al., 2018). Thus,  
75 alternative natural food additives with anti-*Listeria* properties have been  
76 increasingly requested by the food industry for post-processing treatments as this  
77 pathogen has the ability to proliferate under refrigerated conditions, high  
78 concentrations of NaCl and wide pH range(Bucur et al., 2018).

79 Bacteriocins, a heterogeneous group of antimicrobial peptides that are  
80 ribosomally produced by certain bacterial strains (Cotter et al., 2013), are  
81 promising bio-preservatives and antimicrobial agents for the food industry and  
82 medical areas, respectivelyBacteriocins produced by lactic acid bacteria (LAB)  
83 are the most applied in food processes and those present in class IIa are often  
84 described as listericidal (Drider et al., 2006). LAB is known for demanding  
85 numerous amino acids, peptides, nucleic acids and vitamins (Brinques et al.,  
86 2010) leading to, in some cases, their biotechnological use economically  
87 unfeasible, even for high added value biocompound production such as  
88 bacteriocins.

89 The development of alternative growth media using industrial by-products  
90 has been widely studied in order to decrease production costs but also due to  
91 environmental policy trends of circular economy. Cheese whey and vinification  
92 lees (Rodríguez-Pazo et al., 2018), fish wastes (Vázquez et al., 2019), brewer's  
93 spent grain (Paz et al., 2018) and soy whey (Mitra et al., 2010) are some  
94 examples of industrial residues used as substrates for bacteriocin production by  
95 LAB. Considering that the carbohydrate source accounts for 15 to 60% of

96 production costs (Straathof et al., 2011), the use of carbohydrates from  
97 lignocellulosic biomass as renewable raw material has been proposed for  
98 alternative large-scale biotechnological production processes (Du et al., 2012).  
99 An option is sugarcane bagasse, the main lignocellulosic biomass generated by  
100 the sugarcane industry, which has been explored for the production of a broad  
101 group of biotech products due to its high abundance, low cost, renewability and  
102 high fermentable carbohydrate content. Nevertheless, no reports, to the best of  
103 our knowlegde, have been described on the use of industrial sugarcane residues to  
104 produce bacteriocin.

105 Different incremental biomass deconstructive pathways have been used to  
106 break down the lignocellulosic matrix (cellulose, hemicellulose and lignin),  
107 resulting in a lignocellulosic fraction of varying chemical composition (Vieira et  
108 al., 2020, Satlewal et al., 2018). Among the existing fractionation strategies  
109 employed in the lignocellulose liquefaction process, the hydrothermal pre-  
110 treatment has stood out as a promising low-cost and ecological technology  
111 capable of promoting, with short residence times and low sugar degradation  
112 profiles(Haldar et al., 2021). The hydrothermal pretreatment process results in a  
113 hemicellulosic hydrolysate rich in xylooligosaccharides (XOS) (Santucci et al.,  
114 2015) over monomeric sugars, which are mostly obtained in the diluted acid  
115 pretreatment (Roque et al., 2019). *Pediococcus pentosaceus* belongs to the LAB  
116 group and presents an expressive potential in biotechnology industries (Porto et  
117 al., 2017). Studies with this bacterial species have demonstrated a great genetic  
118 diversity related to carbohydrate metabolism (Jiang et al., 2020) as well as the  
119 existence of a number of XOS/arabinoxylan consuming strains (Han et al., 2021,  
120 Jiang et al., 2020).

121 Here, an isolate named *Pediococcus pentosaceus* ET34 (Tomé et al.,  
122 2007) with a potent extracellular listericidal activity (Todorov et al., 2011), was  
123 grown in a culture medium containing non-detoxified hemicellulose hydrolysate.  
124 Many *Pediococcus* strains are described as pediocin PA-1 producers, genome  
125 sequencing of 65 *P. pentosaceus* isolates from different niches revealed that some  
126 strains harbour the operons of other class IIa bacteriocins such as penocin A and  
127 plantaricin (Jiang et al., 2020). Thus, firstly, this work sought to evaluate the  
128 molecular aspects related to bacteriocin production by *P. pentosaceus* ET34.  
129 Secondly, process optimization and preliminary scale up studies were carried out  
130 and ultimately, an integrated industrial-scale process based on biorefinery  
131 practices and the experimental data obtained in the present work was proposed.

132 This study is a pioneer in the use of lignocellulosic residue to increase  
133 bacteriocin production including upstream and downstream production aspects.  
134 In addition, the standardization of a protocol to purify pediocin PA-1 from HSB  
135 containing medium was also explored, which would allow its application in  
136 protocols for pathogen controlling in a precise dose/quantity avoiding the  
137 emergence of bacteriocin-resistant strains. Undoubtedly, the possibility of  
138 producing a pure product with high added value such as pediocin PA-1, using  
139 by-product is a relevant achievement within a vast number of challenges that still  
140 need to be overcome for PA-1 production via circular economy principles.

141

## 142 **2. Materials and Methods**

### 143 **2.1 Bacterial strains**

144 *Pediococcus pentosaceus* ET34 was previously isolated from smoked  
145 salmon (*Salmo salar*) and characterized as bacteriocin producer (Tomé et al.,

146 2007). *Listeria innocua* CLIST 2711 and *L. innocua* DPC3572 strain were used  
147 as bioindicators in the antimicrobial activity assays. Both, *P. pentosaceus* ET34  
148 and *Listeria* strains were cryopreserved at -80°C in their respective media,  
149 supplemented with 20% (v.v<sup>-1</sup>) of glycerol.

150

## 151 **2.2 Microbial growth conditions and culture media**

152 *P. pentosaceus* ET34 was grown at 37 °C under static conditions for 12 to  
153 24 h using MRS-Lactobacilli Broth (Difco Laboratories, Detroit, MI, USA) or  
154 LAPTg medium (Testa de Nadal et al., 1997), without glucose (LAPT: 15g  
155 peptone, 10g yeast extract, 10g tryptone and 0.1% of tween 80). The culture  
156 medium used to optimize pediocin PA-1 production was prepared by mixing a  
157 fixed volume of 2x concentrated LAPT broth (50 % of the final culture volume)  
158 with a variable volume of the hemicellulose hydrolysate of sugarcane bagasse  
159 (HSB) mixed with sufficient sterile deionized water to ensure the remaining 50%.  
160 Cells of *L. innocua* CLIST 2711 or *L. innocua* DPC 3572 for antimicrobial activity  
161 assay inoculum were grown at 37 °C under static conditions for 16 to 24 h in BHI  
162 medium (Difco Laboratories).

163

## 164 **2.3 Genome sequencing and assembly**

165 Genomic DNA of *P. pentosaceus* ET34 was extracted using the Wizard®  
166 Genomic DNA Purification Kit (Promega, Madison, WI, USA) and quantified  
167 using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Paired-end  
168 libraries were prepared using the Nextera XT DNA library preparation kit  
169 (Illumina, San Diego, CA, USA), and sequenced on the MiSeq (Illumina)  
170 platform, using the 600-cycle MiSeq reagent version 3 kit (Illumina). The raw



171 reads were trimmed with Trimmomatic v0.39 using default parameters. The  
172 genome was assembled using the SPAdes pipeline, version 3.14.1 , using the *P.*  
173 *pentosaceus* SRM102736 (CP028259) genome as a guide for repetitive regions  
174 assembly resolution. Genome completeness, contamination and strain  
175 heterogeneity were analyzed via CheckM, version 0.9.6. The Whole Genome  
176 Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession  
177 number JABJXG000000000, and BioProject PRJNA633488. The version  
178 described in this paper is version JABJXG010000000.

179

#### 180 **2.4 Annotation and analysis of genomic data**

181 NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al.,  
182 2018), Rapid Annotation using Subsystem Technology (RAST) Server (Overbeek  
183 et al., 2014), and BlastKOALA (Kanehisa et al., 2016) were used for annotation,  
184 extraction of comprehensive functional information and reconstruction of  
185 metabolic pathways via the Kyoto Encyclopedia of Genes and Genomes (KEGG)  
186 (Kanehisa et al., 2000). The bacteriocin operon was predicted through BAGEL4  
187 pipeline (Kanehisa et al., 2016), and bacteriocin related proteins were identified  
188 through BLASTP analysis (Van Heel et al., 2018). The taxonomic identity of *P.*  
189 *pentosaceus* ET34 strain was confirmed using Average Nucleotide Identity (ANI)  
190 approach by OrthoANI tool (Camacho et al., 2009) using seven *P. pentosaceus*  
191 complete genomes (CP000422, CP046938, CP023655, CP039378, CP028254,  
192 CP028259, and CP023008), *Pediococcus acidilactici* DSM 20284  
193 (NZ\_AEEG01000000) and *Lactococcus lactis* subsp. *lactis* II1403 (AE005176)  
194 as outgroups.

195

## 196 2.5 Pediocin transcript analysis

197 *P. pentosaceus* ET34 was grown on LAPT medium in the presence or  
198 absence of 10% of HSB at 37 °C, and total RNA was extracted after 4, 8 and 24 h  
199 using PureLink RNA Mini kit (Thermo Fisher Scientific Inc., Waltham, MA,  
200 USA). The integrity of the total RNA was checked on an agarose gel (1.0%) and  
201 quantified by absorbance (260 nm) using a NanoDrop ND-1000  
202 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All the  
203 materials used were sterilized and later treated with RNaseZAP™ (Thermo Fisher  
204 Scientific) prepared with diethyl pyrocarbonate-treated water to prevent nucleic  
205 acid degradation. Residual DNA was removed using DNase Purelink (Thermo  
206 Fisher Scientific). Retrotranscription from mRNA to cDNA was performed with  
207 SuperScript™ First-Strand following the manufacturer's recommendations.

208 The amplification reaction was performed separately for each gene in a  
209 final volume of 20 µL, containing 5 µL of cDNA (1 ng for *pedA*, *pedC*, *pedD* and  
210 0.01 ng for 16S gene, respectively), 0.3 µL of 10 µM of each oligonucleotide and  
211 10 µL of the Power SYBR® Green qPCR Master Mix solution (Thermo Fisher  
212 Scientific). For each condition tested, technical duplicates of a total of three  
213 biological replicates were analyzed. Real time PCR was performed on a StepOne  
214 Real-time PCR System (Thermo Fisher Scientific) using an amplification  
215 program consisting of an initial denaturation at 95°C for 10 min, followed by 40  
216 cycles of amplification at 95°C for 15 s and 60°C for 1 min, and a melting curve  
217 analysis (95°C for 1 min, cooling to 60°C for 15 s and a final ramp to 95°C at a  
218 rate of 0.3°C·s<sup>-1</sup>).

219 The relative expression ratio (*R*) was quantified according to the equation  
220 1, described by Pfaffl et al., (2001).

221

$$222 \quad R = \frac{E_{(target)}^{\Delta C_{Ptarget}(control-sample)}}{E_{(ref)}^{\Delta C_{Pref}(control-sample)}} \quad (\text{eq. 1})$$

223

224         The evaluated target gene were those related to pediocin synthesis (*pedA*,  
225 *pedC* and *pedD*) and the reference gene the 16S rRNA, as previously described  
226 by Fernandez et al., (2014) *P. pentosaceus* ET34 cultured in LAPT medium  
227 supplemented with 10% HSB was taken as the test sample, and *P. pentosaceus*  
228 ET34 grown in LAPT medium was included as the control. PCR products of  
229 target and reference samples were considered to double (PCR efficiency, E=2) in  
230 each cycle. Statistical significance analysis of the obtained data was performed  
231 using the Minitab 17 Statistical Software (State College, PA, USA: Minitab, Inc.,  
232 www.minitab.com).

233

## 234 **2.6 Antimicrobial activity determination**

235         To evaluate bacteriocin production, aliquots of culture medium were  
236 collected and centrifuged at 3000 g, 25 °C for 10 min. To prevent the  
237 antimicrobial effect of organic acids, the pH was adjusted to 6.0-6.5 using 1.0 N  
238 NaOH. To inactivate extracellular proteases, the samples were heated at 80°C for  
239 10 min and then filtered to give *P. pentosaceus* ET34 cell free supernatant (CFS).  
240 The CFS antimicrobial activity, expressed in arbitrary units (AU) per mL, was  
241 quantified indirectly by the drop-agar diffusion method (Sabo et al., 2018) using  
242 equation 2:

$$243 \quad \frac{AU}{mL} = \frac{\pi r^2}{v} \quad (\text{eq. 2})$$

244

245 where  $r$  is the radius of the inhibition halo (mm) and  $v$  the volume (mL) of CFS  
246 applied to Petri plates. Briefly, 20  $\mu$ L of CFS were pipetted onto the surface of  
247 freshly prepared solid BHI culture medium (0.8% agar) containing *L. innocua*  
248 CLIST 2711 ( $\sim 10^6$  CFU). After absorption, the plates were incubated at 37 °C for  
249 24 h, and the inhibition halos measured using a high precision electronic digital  
250 Vernier caliper.

251

## 252 2.7 Preparation of non-detoxified hemicellulose hydrolysate

253 The HSB was obtained by hydrothermal pre-treatment of sugarcane  
254 bagasse. Biomass was provided by Ferrari Agroindustrial (Pirassununga, SP,  
255 Brazil) and used without comminution and previous washing. The chemical  
256 composition of the dry raw material, determined according to Sluiter et al.,  
257 (2008), was  $43.02 \pm 0.34\%$  cellulose,  $25.46 \pm 0.12\%$  hemicellulose,  $22.32 \pm$   
258  $0.43\%$  lignin,  $4.62 \pm 0.25\%$  ash, and  $4.23 \pm 0.11\%$  extractives.

259 The hydrothermal pretreatment reaction was performed in the Pilot Plant  
260 for Process Development at the Brazilian Biorenewables National Laboratory  
261 (LNBR/CNPEM/MCTIC) (Campinas, SP, Brazil). Around 20 kg of raw  
262 sugarcane bagasse with 50% ( $w \cdot w^{-1}$ ) moisture content were fed into a 350-L  
263 alloy steel reactor (Pope Scientific Inc, Saukville, WI, USA) with 10% ( $w \cdot w^{-1}$ )  
264 solids loading, and the reaction was performed at 190 °C for 10 min, according to  
265 Santucci et al. (2015). Thermal fluid was percolated through the jacket heating  
266 reactor, which was continuously stirred at 150 rpm. After completion of the  
267 reaction, the fermenter was cooled, depressurized, and opened. The solid and  
268 liquid fractions were separated with a Nutsche filter (Pope Scientific Inc.) with  
269 140 L capacity. The solid fraction was stored in a cold chamber (18 °C) and used

270 in other studies, while the liquid fraction, HSB, was concentrated four times in a  
271 wiped film evaporator (Pope Scientific Inc.), with up to 50 kg·h<sup>-1</sup> of water  
272 evaporation capacity at 80 °C and 470 mbar.

273 The resulting concentrate was stored in a cold container for further  
274 fermentation studies. Prior to use for the alternative culture medium formulation,  
275 a batch of crude HSB was adjusted to pH 6.5, and the resulting precipitate  
276 (phenolic compounds from lignin) removed by centrifugation at 6,690 g for 20  
277 min.

278 The composition of the resulting liquid fraction was then determined by  
279 different analytical methods. Monosaccharides (glucose, xylose, and arabinose),  
280 cellobiose, acetic, levulinic and formic acids were analyzed using a 1260 Infinity  
281 HPLC system (Thermo Fisher Scientific) equipped with a refractive index  
282 detector and fitted with an analytical Aminex HPX-87H column (300 mm × 7.8  
283 mm, 9 µm) in combination with a guard column consisting of Micro-Guard  
284 Cation PC H Refill Cartridges (Bio-Rad Laboratories Inc., Hercules, CA, USA).  
285 The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.6 mL·min<sup>-1</sup>.  
286 For furfural and hydroxymethylfurfural (HMF) quantification, a reversed-phase  
287 HPLC fitted with an Acclaim 120 C18 column (150 mm × 4.6 mm, 3 µm) and a  
288 single wavelength UV detector were employed. The mobile phase consisted of a  
289 mixture of water-acetonitrile 1:8 (v·v<sup>-1</sup>) with 1% acetic acid (v·v<sup>-1</sup>) at a flow rate  
290 of 0.8 mL·min<sup>-1</sup>.

291 Xylooligosaccharides (XOS) were determined according to Mok and Antal  
292 (1992). A quantitative post-hydrolysis with 4% (w·w<sup>-1</sup>) sulfuric acid at 120 °C  
293 for 1 h was performed. The XOS content was calculated as the difference  
294 between the monosaccharide content measured via HPLC before and after acid

295 hydrolysis, considering in the mass balance the formed degradation/solubilization  
296 products (furfural, HMF, levulinic, acetic and formic acids).

297

## 298 **2.8 Experimental design and analysis**

299 To maximize pediocin production by *P. pentosaceus* ET34 using HSB as  
300 an alternative low-cost renewable carbon source, a Response Surface  
301 Methodology (RSM) based on Central Composite Design (CCD) (Bhattacharya,  
302 2021), was performed, considering temperature and % of HSB as variables.

303 RSM is a collection of mathematical and statistical techniques used in the  
304 development of an adequate functional relationship to optimize a response  
305 (output variable) that is influenced by several independent variables (input  
306 variables) (Bhattacharya, 2021), employing a smaller number of assays, which  
307 can lead to a costs reduction to maximize or minimize some operational response,  
308 in our case the antimicrobial activity.

309 The analysis of the results includes the computation of the linear (L),  
310 quadratic (Q), and interaction effects, and the analyses of the variances ascribed  
311 to them. The statistical significance of these effects was evaluated by the analysis  
312 of variance using the *F* test (Rabelo et al., 2008). A quadratic model was obtained  
313 relating each response variable to the significant effects. These models were used  
314 to define the conditions that separately and simultaneously maximize the  
315 response variable. The Statistica 7.0 software (Statsoft, Inc., Tulsa, OK, USA)  
316 was used to implement all analyses, and the result was illustrated by RSM.

317 Cells of *P. pentosaceus* ET34 used for the inoculum in the different runs  
318 were grown for 12 h after the addition of 100  $\mu$ L of stock culture into 50 mL of  
319 commercial MRS broth in Falcon tube. Biomass was then centrifuged at 2,860 g

320 for 5 min at 25 °C (Sorvall Lynx 4000, Thermo Fisher Scientific) and washed  
321 with saline solution (0.85% NaCl). Based on McFarland standard, the biomass  
322 pellet was resuspended at a concentration of  $10^7$  CFU·mL<sup>-1</sup>. After 14 h of  
323 cultivation, the CFS obtained in each run had its antimicrobial activity evaluated  
324 as previously described.

325

## 326 **2.9 Scale-up of pediocin PA-1 production**

327 Using the optimized operational conditions optimized, the production of  
328 pediocin PA-1 was then evaluated at larger-scale under static conditions in a 3-L  
329 Erlenmeyer flask containing 2 L of LAPT + 20.6% HSB culture medium, whose  
330 carbon source composition (g·L<sup>-1</sup>) was glucose, 0.25; xylose, 15.94; arabinose,  
331 17.78; cello-oligosaccharides, 3.42; XOS, 43.85; and arabinose oligomers, 6.35.  
332 The concentrations of carbon sources, including those in oligomeric form, lactic  
333 and acetic acid, HMF and furfural, were determined over time, as described  
334 before. During cultivations, carried out in triplicate, samples were collected every  
335 2 h during the first 14 h and at the end (24 h), and the antimicrobial activity was  
336 determined as previously described. All the chemical analyses were performed in  
337 triplicate.

338

## 339 **2.10 Bacteriocin purification**

340 *P. pentosaceus* ET34 pediocin PA-1 was purified from CFS by a 4-step  
341 process using Amberlite XAD16N hydrophobic resin, SP Sepharose Cation  
342 exchange resin, C18 SPE and Reversed phase HPLC. The presence of pediocin  
343 PA-1 at each purification step was confirmed indirectly by antimicrobial activity  
344 against *L. innocua* DPC3572 and directly by MALDI-TOF MS spectrometry

345 (Axima TOF<sup>2</sup>, Shimadzu Biotech, Wolverton, MK, UK). In the first purification  
346 step, the pediocin PA-1-containing CFS was applied to a column containing  
347 Amberlite XAD16N resin (Sigma-Aldrich, Saint Louis, MO, USA). After  
348 adsorption, the resin was washed with a 20% ethanol solution, and pediocin PA-1  
349 eluted from the column using 70% isopropanol (Sigma-Aldrich) containing 0.1%  
350 trifluoroacetic acid (TFA) (Sigma-Aldrich), named 70 IPA. The alcohol was  
351 evaporated from the 70 IPA eluent by rotary evaporation (Büchi Labortechnik  
352 AG, Flawil, Switzerland), and the sample pH was adjusted to 4.4 with 1N NaOH  
353 to optimize binding to the SP Sepharose cation exchange column (second  
354 purification step). After absorption, the SP sepharose resin was washed with 20  
355 mM sodium acetate at pH 4.4 and 20 mM sodium acetate at pH 4.4 containing  
356 150 mM NaCl to remove impurities, and pediocin PA-1 was then eluted with 20  
357 mM of sodium acetate at pH 4.4 containing 1 M NaCl. In the third purification  
358 step, NaCl was removed using a C18 SPE cartridge, and the washing and elution  
359 steps were performed as described for the first purification step.

360 Finally, the bacteriocin was purified to homogeneity by Reversed Phase  
361 HPLC using a C12 reverse phase Jupiter Proteo column (250 x 10.0 mm, 4 µm  
362 90Å) running a 25-40% acetonitrile, 0.1% TFA gradient where mobile phase A  
363 was 0.1% TFA and mobile phase B 100% acetonitrile with 0.1% TFA. Eluted  
364 fractions containing pediocin PA-1 were identified by detection of molecular  
365 mass (~4620-4625 Da) using MALDI-TOF MS, and pure fractions were pooled  
366 and lyophilized using a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswich,  
367 UK).

368

### 369 **3. Results and Discussion**



### 370 3.1 Genomic analysis

371 Genome sequencing of *Pediococcus pentosaceus* ET34, isolated from food  
372 vacuum-packed-cold-smoked salmon (Tomé et al., 2007), yielded a total of  
373 2,531,195 high-quality paired-end reads showing a PHRED score above 32. They  
374 were assembled in 19 contigs showing N50 of 443-kb and ~80x of coverage,  
375 resulting in a draft genome sequence of 1,953,235 bp with an average of 37.7%  
376 G+C. CheckM analysis revealed completeness of 99.38%, showing no  
377 contamination. According to the PGAP annotation pipeline, the strain harbors  
378 1,883 coding genes, 57 tRNAs, 3 ncRNAs, and five copies of the ribosomal  
379 operon. Moreover, 50 pseudogenes were identified. An ANI heatmap matrix was  
380 built based on the sequences of species closest to the isolate (Figure 1a).

381 It was possible to confirm previous identification (Tomé et al., 2008) of *P.*  
382 *pentosaceus* ET34 identity at species level with *P. pentosaceus* (ANI value >  
383 98.6%). Based on ANI values, a high similarity was evident between *P.*  
384 *pentosaceus* ET34 and strains isolated from food, i.e., *P. pentosaceus*  
385 SRCM102736 from soybean paste and *P. pentosaceus* JQI-7 from fermented  
386 dairy product. Up to 1,774 CDS were annotated with KEGG Orthology (KO) and  
387 Enzyme commission number (EC). BAGEL4 revealed that the *P. pentosaceus*  
388 ET34 genome contained the complete pediocin PA-1 operon located in proximity  
389 to a number of plasmid-related genes (replication protein and *traA*) (Figure 1b),  
390 suggesting that the pediocin operon is present on a plasmid, as previously  
391 observed for many other *Pediococcus* spp (Papagianni and Anastasiadou et al.,  
392 2009).  
393

394 **3.2 Pediocin PA-1 production and relative genes expression by *P. pentosaceus***  
395 **ET34 in the presence of hydrolysate of sugarcane bagasse**

396 To evaluate the biotechnological potential of using hydrolysate of sugarcane  
397 bagasse for pediocin PA-1 production by *P. pentosaceus* ET34, a glucose-free  
398 adapted LAPT broth formula supplemented with HSB was prepared. Given the  
399 lack of existing knowledge about the tolerance of *P. pentosaceus* ET34 to  
400 inhibitory compounds formed and concentrated during HSB production and  
401 processing, e.g., furfural and hydroxymethylfurfural (HMF), the initial set of  
402 experiments to evaluate pediocin PA-1 production and pediocin PA1-related gene  
403 expression was performed using 10% HSB. Aside from the primary carbon  
404 source, the adapted culture medium had a similar composition to that normally  
405 used for bacteriocin production by LAB (Juarez Tomás et al., 2002). Both,  
406 LAPT medium with 10% HSB and without HSB (control) were inoculated with  
407 an initial viable cell count of approximately  $10^7$  CFU·mL<sup>-1</sup> and incubated at pH  
408 6.0 and 37 °C for 31 h. Figure 2a shows the *P. pentosaceus* ET34 growth and the  
409 variation over cultivation time in antimicrobial activity ( $\Delta$  antimicrobial activity),  
410 i.e., the difference between the inhibitory effects of LAPT + 10% HSB and  
411 control cell free supernatants (CFS) against *L. innocua* CLIST 2711.

412 Since cell viability was not adversely affected by HSB toxicity and anti-  
413 *Listeria* activity was improved compared to the control run, it was possible to  
414 conclude that the selected HSB percentage provided suitable conditions for *P.*  
415 *pentosaceus* ET34 growth and greatly improved pediocin PA-1 production. The  
416 CFS anti-*Listeria* activity progressively increased throughout microbial growth  
417 reaching a maximum value of  $\sim 6,500$  AU·mL<sup>-1</sup> after 8 h of cultivation. After  
418 remaining constant for up to 14 h, the variation in anti-*Listeria* activity decreased

419 between 15 and 31 h, probably due to the proteolytic action of extracellular  
420 proteases produced by *P. pentosaceus* ET34.

421 To evaluate if the expression of related pediocin PA-1 genes was increased  
422 in the presence of HSB, relative expression analysis of pediocin structural  
423 (*pedA*), accessory (*pedC*) and transporter (*pedD*) genes was performed on  
424 samples collected after 4, 8 and 24 h from both fermented media. Figure 2b  
425 shows that the relative expression of *pedA*, *pedC* and *pedD* during *P.*  
426 *pentosaceus* ET34 growth in LAPT medium supplemented with 10% HSB was  
427 up-regulated after all tested times, with the highest CFS anti-*Listeria* activity  
428 detected after 8 h.

429 Notably, among the pediocin PA1-related transcripts, *pedD* showed the  
430 most pronounced relative expression, consistent with the observations of  
431 Fernandez et al., (2014). These results are also in line with the observations  
432 made for *P. acidilactici* (Kim et al., 2018) in the presence of inulin  
433 nanoparticles, whose *pedA* and *pedD* genes were up-regulated compared to  
434 controls, and in which the presence of the prebiotic also triggered the expression  
435 of genes associated with stress responses, i.e., *groEL*, *groES*, *dnaK41*.

436 Heterologous expression of pediocin genes in *Escherichia coli* revealed  
437 that only *pedA* and *pedD* are essential for pediocin production in its active form  
438 (Mesa-Pereira et al., 2018). In this recombinant system, *pedC*, an accessory  
439 protein involved in the formation of disulphide bonds, just increased pediocin  
440 PA-1 production. Although more in-depth studies are needed to evaluate the  
441 exact consequences of up-regulating pediocin PA-1 production by *P. pentosaceus*  
442 ET34, one hypothesis could be that the presence of toxic substances in the HSB

443 and/or the prebiotic action of xylooligosaccharides (XOS) may have up-regulated  
444 pediocin PA-1 gene expression via stress-related mechanisms.

445

### 446 3.3 Pediocin purification

447 Although the analysis of mRNA transcripts showed a significant increase  
448 in the relative expression of the ratio of pediocin PA-1 *pedA*, *pedC*, and *pedD*  
449 genes in the presence of HSB, a sequence of purification steps was employed to  
450 isolate pediocin PA-1 and analytically confirm the strain ability to produce such  
451 bacteriocin. Given the decrease in antimicrobial activity previously observed  
452 after 15 h of fermentation, a new CFS was prepared by growing the strain at 37  
453 °C for 14 h in in LAPT medium supplemented with 10% HSB.

454 Pediocin PA-1 partially purified from CFS according to a non-optimized  
455 4-step protocol and eluents at the end of each purification step were assayed for  
456 antimicrobial activity against *L. innocua* DPC3572. Samples tested positive for  
457 anti-*Listeria* activity were then analyzed by MALDI-TOF MS to assess their  
458 relative purity (Figure 3).

459 MALDI-TOF MS of the 70 IPA eluent from the first purification step  
460 detected a molecular mass of 4625 Da corresponding to that of pediocin PA-1  
461 (4624 Da), although several other molecular masses were also detected  
462 suggesting that the sample was impure (Figure 3a). This eluent was then further  
463 purified by cation ion exchange, and MS analysis of the active 1 M NaCl-  
464 containing eluent revealed a mass corresponding to that of pediocin PA-1 (Figure  
465 3b). Again, the presence of other masses suggested that this sample still  
466 contained impurities. Therefore, salt was removed from the pediocin PA-1-  
467 containing eluent by applying it to a C18 SPE column, and MALDI-TOF MS

468 analysis showed an increased peak corresponding to pediocin PA-1 mass compared to  
469 those of contaminants, suggesting an increase in the relative bacteriocin concentration  
470 (Figure 3c).

471 Finally, reversed phase HPLC was used to purify the sample to >95%  
472 purity, and HPLC fractions deemed pure by MALDI-TOF MS (Figure 3d) were  
473 pooled and lyophilized to give 0.8 mg L<sup>-1</sup> of culture medium. Although, the  
474 purification protocol was adopted simply to validate the production of pediocin  
475 PA-1 by *P. pentosaceus* ET34, it proved suitable for purifying this bacteriocin  
476 from a complex culture medium to a high purity degree. As the purification  
477 protocol used was generic and not optimized for pediocin PA-1, some of the  
478 bacteriocin was lost in the column washing steps during purification, meaning  
479 there is potential to further improve yield.

480

### 481 **3.4 Optimization of small-scale cultivation conditions**

482 The temperature and percentage of HSB in the medium were selected as  
483 the independent variables to maximize small-scale pediocin PA-1 production by  
484 *P. pentosaceus* ET34. Depending on the operating conditions, antimicrobial  
485 activity ranged from 4142 to 14,464 AU·mL<sup>-1</sup> and peaked at 34 °C and 22.1%  
486 HSB.

487 Figure 4a shows the Pareto chart of standardized effects, considering the  
488 significant effects at 90% level of significance. It is possible to see that the largest  
489 effect was the linear one of %HSB, followed by the linear effect of temperature,  
490 both positively influencing the antimicrobial activity. In general, the interaction  
491 factors had less significant effects on the antimicrobial activity, and all effects  
492 (linear, quadratic, and interaction factors) were significant.

493 Table 1 depicts the analysis of variance (ANOVA) for the model of  
494 antimicrobial activity. It can be seen that the model exhibited a high correlation  
495 coefficient and can be considered statistically significant with 90% of confidence  
496 according to the F-test, as it presented calculated F-values greater than the listed  
497 ones (Rabelo et al., 2008). Also, it do not present evidence of lack of fit, as the  
498 calculated values by the F-test for the lack of fit were much smaller than the  
499 listed values. Therefore, the experimental data were accurately fitted by the  
500 quadratic model represented by Equation 3:

501  
502 **Antimicrobial activity**  $\left(\frac{\text{AU}}{\text{mL}}\right) = 2988.96 C - 840.15 C^2 + 2003.83 T - 634.95 T^2 -$   
503  $619.67 CT + 11231.67$  (Eq. 3)

504 where C and T are the coded values of the HSB percentage and the temperature,  
505 respectively.

506

507 The proposed quadratic model (Eq. 3) was then used to plot the response  
508 surface (Figure 4b) and for optimization, purposes select the operating conditions  
509 capable of maximizing the response. The temperature of 36.8 °C and 20.6% HSB  
510 in the medium were identified as the optimal conditions, with an estimated  
511 antimicrobial activity of 14,132 AU·mL<sup>-1</sup> (Figure 4b). An independent  
512 experiment performed under the predicted optimal conditions confirmed that they  
513 ensured an anti-*Listeria* activity almost coincident with that achieved in the run  
514 number 6 (14,465 AU mL<sup>-1</sup>), thereby validating the predictability of the selected  
515 model.

516 The result of the optimization run was in line with those performed for  
517 relative genes expression evaluation, in which pediocin-related transcripts were  
518 up-regulated. Moreover, it was corroborated by observations made in previous

519 studies, where bacteriocin production was described as associated with defense  
520 mechanisms subject to the effects of pH, pressure, temperature, O<sub>2</sub> concentration,  
521 and cell density (Papagianni and Anastasiadou, 2009). In this sense, it is possible  
522 to infer that the simultaneous increase in both variables could have triggered  
523 internal cell control mechanisms related to bacterial stress.

524

### 525 **3.5 Pediocin PA-1 production scale-up**

526 To highlight possible scale-up effects on bacteriocin production, Figure 5  
527 shows lactic acid concentration, *P. pentosaceus* ET34 CFS anti-*Listeria* activity  
528 and concentrations of carbon sources over time during cultivation carried out in  
529 3-L Erlenmeyer flask under static conditions (Figure 5a). Lactic acid production  
530 was detected only after 4 h of cultivation (1.23 g.L<sup>-1</sup>), increased up to a maximum  
531 value of approximately 3 g.L<sup>-1</sup> after 8 h, and remained almost constant until the  
532 end of the run ( $p > 0.05$  between 8 and 24 h). Given the growing interest in lactic  
533 acid as a future green key feedstock in the production of a broad group of  
534 chemicals like oxygenated compounds (e.g., acrylic acid), green solvents (e.g.,  
535 2,3-pentanedione) and bioplastics (e.g., poly-lactic acid), its use could be  
536 considered from a biorefinery perspective.

537 The CFS anti-*Listeria* activity was first detected after 6 h of cultivation  
538 (3451 AU.mL<sup>-1</sup>) and then increased progressively up to 9963 AU.mL<sup>-1</sup> after 24 h.  
539 Although *P. pentosaceus* is a homofermentative lactic acid-producing bacterium,  
540 a small production of acetic acid was observed during *P. pentosaceus* ET34  
541 growth (from 1.67 to 2.2 g.L<sup>-1</sup>), as a likely result of arabinose metabolization  
542 following glucose depletion after approximately 4 h (Figure 5b). Indeed,  
543 arabinose consumption occurs likely due to the activity of citrate metabolism

544 enzymes, e.g., phosphotransacetylase (EC 2.3.1.8) and acetate kinase encoded  
545 within the *P. pentosaceus* ET34 genome. Of the different carbon sources  
546 available, *P. pentosaceus* ET34 was only able to consume the free glucose and  
547 arabinose present in the culture medium, while concentrations of XOS,  
548 arabinoxylan and monomeric xylose remained constant up to the end of  
549 cultivation. The presence of genes encoding enzymes involved in arabinose and  
550 glucose consumption, e.g., L-arabinose isomerase (EC 5.3.1.4) and L-ribulose  
551 phosphatase 4-epimerase (EC 5.1.3.4), as well as in glycolysis and pentose  
552 phosphate pathway found in the *P. pentosaceus* ET34 genome is consistent with  
553 the observations made on carbon source consumption.

554         Although the ability to consume prebiotics such as XOS is a characteristic  
555 of only a small group of LAB strains, the presence of genes encoding XOS  
556 transportation proteins and degrading enzymes (e.g., arabinofuranosidase and  
557 xylanases) has been identified in different *P. pentosaceus* strains (Lei et al.,  
558 2018). The absence of genes encoding enzymes related to xylose metabolisms as  
559 well as XOS and arabinoxylan hydrolysis [e.g.,  $\alpha$ -1,2- arabinofuranosidase (EC  
560 3.2.1.55), 1,4- $\beta$ -xylosidase (EC 3.2.1.37), endo-1,4- $\beta$ -xylanase (EC 3.2.1.8),  
561 xylose isomerase (EC 5.3.1.5), D-xylose-reductase (EC 1.1.1.307), D-xylulose  
562 reductase (EC 1.1.1.9)] is consistent with the inability of *P. pentosaceus* ET34 to  
563 consume xylose and arabinoxylans. The final anti-*Listeria* activity was found to  
564 be 1.45 times lower than that detected in the early stages of the bioprocess.

565         Since the predicted optimum conditions were experimentally validated at  
566 small scale, the lower performance of *P. pentosaceus* ET34 in the scaled-up run  
567 may be ascribed to a combination of detrimental factors affecting the interactions  
568 between the cellular machinery and the extracellular environment (e.g.,



569 heterogeneity in a larger volume, absence of mixing under static conditions, and  
570 mass transfer limitations). While satisfactory for early bioprocess  
571 development, multi-scale analysis should be considered when using scale-up  
572 cultivation systems in order to perform a reliable future industrial pediocin  
573 PA-1 production process.

574

### 575 **3.6 Pediocin PA-1 integrated production process**

576 Composed of cellulose, hemicellulose, and lignin, as well as other minor  
577 constituents, sugarcane bagasse is considered the most abundant byproduct of the  
578 sugar and alcohol agroindustry. Given its origin and renewable character, such a  
579 biomass stands out as a cheaper alternative to conventional sugar sources (Østby  
580 et al., 2020), without entering into direct competition with food chain and/or  
581 affecting arable land management. Herein, an integrated process is proposed that  
582 covers the application of expanded conversion platforms based on the  
583 concomitant transformation of sugarcane bagasse into a vast range of products  
584 and co-products, focused on the biotechnological production of pediocin PA-1  
585 (Figure 6).

586 Given the importance of recycling hazardous volatile organic solvents and  
587 reducing waste produced during the development of greener and more sustainable  
588 processes (Welton, 2015), such aspects were also taken into account in the  
589 assessment of environmental friendliness and economic viability of future  
590 processes. The unit steps explored experimentally in the present work are  
591 represented in green and include: (i) sugarcane bagasse pre-treatment, (ii)  
592 hydrolysis of the hemicellulose fraction, (iii) pediocin PA-1 production, (iv)  
593 downstream processing, and (v) polishing.

594 The proposed additional steps are depicted in dashed lines and include:  
595 lignin recovery, cellulose hydrolysis and bioethanol production (in yellow), as  
596 well as arabinoxylan hydrolysis, production of acetic acid, furfural and HMF,  
597 recovery of xylose-rich streams for further chemical and/or bioconversion (in  
598 pink). A loading of 20 kg of raw sugarcane bagasse composed, on a dry basis, of  
599 ( $w \cdot w^{-1}$ )  $43.03 \pm 0.33\%$  cellulose,  $25.46 \pm 0.21\%$  hemicellulose,  $16.81 \pm 0.12\%$   
600 lignin,  $15.23 \pm 0.22\%$  extractives, and  $1.18 \pm 0.09\%$  ash [50% ( $w \cdot w^{-1}$ ) moisture  
601 content] was subjected to a hydrothermal pre-treatment reaction at  $190^{\circ}\text{C}$  for 10  
602 min.

603 After the initial biomass processing, the obtained solid and liquid fractions  
604 were separated by a Nutsche filter ( $69.9 \pm 2.5\%$  of pretreatment yield). The  
605 cellulignin, solid fraction obtained after pretreatment was composed, on a dry  
606 basis ( $w \cdot w^{-1}$ ) of  $57.50 \pm 1.11\%$  cellulose,  $15.50 \pm 0.89\%$  hemicellulose,  $24.05 \pm$   
607  $0.44\%$  lignin, and  $1.00 \pm 0.22\%$  ashes. Insights from the literature can be used for  
608 its exploitation. For instance, a first processing step based on the enzymatic  
609 hydrolysis was substantiated using cellulase cocktails (Zhuang et al., 2015).  
610 Lignin was recovered by filtration for further conversion into chemicals and/or  
611 different biomaterials. Furthermore, the obtained cellulose hydrolysate was used  
612 for the productions of second generation bioethanol (Abdeshahian et al., 2020) or  
613 different high added value biocompounds (Dietrich et al., 2019). The filtered  
614 hemicellulose hydrolysate, composed of  $0.23 \text{ g} \cdot \text{L}^{-1}$  glucose,  $4.22 \text{ g} \cdot \text{L}^{-1}$  cello-  
615 oligosaccharides,  $6.57 \text{ g} \cdot \text{L}^{-1}$  xylose,  $1.03 \text{ g} \cdot \text{L}^{-1}$  arabinose,  $22.93 \text{ g} \cdot \text{L}^{-1}$   
616 arabinoxylan,  $0.55 \text{ g} \cdot \text{L}^{-1}$  formic acid,  $0.09 \text{ g} \cdot \text{L}^{-1}$  HMF and  $1.10 \text{ g} \cdot \text{L}^{-1}$  free acetic  
617 acid may then be mixed with LAPT culture medium and further used.

618 The production of pediocin PA-1 can be conceived through microbial  
619 cultivation employing two possible alternative approaches: in the former  
620 approach *P. pentosaceus* ET34 would be used in pure culture, even considering  
621 its inability to consume xylose and arabinoxylans, while in the latter in co-culture  
622 with other pediocin-producing strains capable of consuming different carbon  
623 sources. The latter approach aimed at increasing pediocin PA-1 production would  
624 be based on synergistic microbial interactions attributable to competition among  
625 strains for nutritional resources. Such synergism was previously demonstrated for  
626 a co-culture of *P. pentosaceus* 147 and *Lactobacillus plantarum* LE27 in cheese  
627 whey broth, which showed a significant increase in bacteriocin production  
628 compared with cultivations performed employing pure cultures (Gutiérrez-Cortés  
629 et al., 2018).

630 Such effects were corroborated by the presence of *pedB* gene located in  
631 the pediocin PA-1 operon of pediocin-producing strains, which confers self-  
632 immunity to this membrane-disrupting bacteriocin and allows their growth in  
633 mixed microbial cultures. A large genome study on 65 *P. pentosaceus* strains  
634 revealed the presence of putative operons involved in different carbohydrate  
635 transport and utilization among strains as well as the presence of a pediocin PA-1  
636 operon in 12 of them (Jiang et al., 2020).

637 A pediocin PA-1 pre-purification/concentration step using a cross-flow  
638 ultrafiltration system with membranes of different cut-offs can be proposed as a  
639 first downstream step. Outlet streams containing pediocin PA-1 would be filtered  
640 after biomass separation using a 10 kDa cut-off membrane to eliminate high  
641 molecular weight contaminants present in the fermented medium.

642           Considering a pediocin PA-1 molecular weight of approximately 4.6 kDa,  
643 the obtained permeate could be subject to a second filtration step using a 2.0 kDa  
644 cut-off membrane, in order to perform bacteriocin concentration and elimination  
645 of the main low molecular weight contaminants. The concentrated pediocin PA-1  
646 (retentate) could finally be purified by a 4-step sequential chromatographic  
647 protocol using a) a hydrophobic interaction column containing XAD resin, b) a  
648 Sepharose cation exchange column, c) a C18 solid phase extraction cartridge, and  
649 d) a C12 reverse phase HPLC column, as previously shown in the present study.  
650 To enhance its stability over time, the pediocin PA-1 could then be lyophilized.

651           Given the high hemicellulose content of final permeate streams obtained  
652 by fractionation of pediocin PA-1 according to the first protocol proposed for  
653 bacteriocin production (pure *P. pentosaceus* ET34 cultivation process), two  
654 different approaches can be proposed: a) a conversion platform based on  
655 processes reported in the literature to produce different valuable co-  
656 products/building blocks from non-metabolized xylose and arabinoxylans (i.e.,  
657 biphasic hydrolysis and dehydration, aldol condensation, furfural–acetone–furfural  
658 (FAF) hydrogenation, and H-FAF hydrodeoxygenation) (Olcay et al., 2018), and  
659 b) an alternative approach aimed at using recovered raw arabinoxylans as  
660 prebiotics in food and feed formulations.

661           The retentate obtained after filtration (using a 10 kDa cut-off membrane)  
662 could be submitted to a polishing step, and the recovered arabinoxylans finally  
663 lyophilized. As regards the volatile organic solvents to be used in the previously  
664 proposed operations/reactions, well-established recycling processes can be  
665 envisaged, i.e., isopropanol pervaporation (Urriaga et al., 2006) and THF  
666 distillation.

667 Urtiaga et al., (2006) described the recovery of isopropanol from industrial  
668 waste (mainly composed of a water/isopropanol mixture) through the  
669 pervaporation process proposed herein, as well as the recycling of THF with a  
670 purity degree greater than 96% using a distillation column (40 stages, SS6Mo). It  
671 is believed that the proposed integrated process could be applied in a sugarcane  
672 biorefinery context as part of a future biomass valorization pathway, especially in  
673 countries with a large sugarcane production such as Brazil and India.

674

#### 675 **4. Conclusions**

676 Pediocin application in industry requires overcoming two bottlenecks:  
677 evidence of safety and efficacy required by regulatory agencies, and reduction of  
678 pure pediocin PA-1 production costs, which is currently economically unfeasible.  
679 In this work, molecular data, process optimization and scale-up have shown that  
680 HSB induces the expression of pediocin PA-1-related genes and can be used as a  
681 carbon source for ET34 growth. An integrated process that includes from the  
682 pretreatment of sugarcane bagasse to the obtaining of pure PA-1 paves the way  
683 for the production of this high added-value compound with green biorefinery  
684 concepts and practices to reduce process costs.

685

686 E-supplementary data of this work can be found in online version of the paper

687

#### 688 **Conflicts of interest**

689 There are no conflicts of interest to declare.

690

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699

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897 **Figure captions**

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899 **Figure 1.** OrthoANI heatmap (a) showing the average nucleotide identity of  
900 *Pediococcus pentosaceus* ET34 with seven *P. pentosaceus* complete genomes and  
901 *Lactococcus lactis* subsp. *lactis* II1403 (SAMN02603339) genomes were used as  
902 outgroups. In b, schematic representation of the bacteriocin operon. The pediocin PA-1  
903 gene cassette consists of four contiguous genes, namely *pedA*, *pedB*, *pedC*, *pedD*,  
904 which encode structural, immunity protein, accessory protein, and transporter,  
905 respectively.

906

907 **Figure 2.** *Pediococcus pentosaceus* ET34 growth and variation of antimicrobial activity  
908 determined over the cultivation time at 37 °C without stirring for 31 h (a). The variation  
909 of antimicrobial activity was defined as the difference between the inhibitory effect of  
910 cell free supernatant of fermented LAPT + 10% HSB medium and the control, assessed  
911 against *L. innocua* CLIST 2711. In b, relative gene expression of *pedA* (yellow), *pedC*  
912 (red), *pedD* (green) of *P. pentosaceus* ET34 cells grown in LAPT medium  
913 supplemented with 10% HBC (test sample) and *P. pentosaceus* ET34 grown only in  
914 LAPT medium (control). Samples were collected after 4, 8 and 24 h.

915

916 **Figure 3.** Mass spectra of the fractions obtained from *Pediococcus pentosaceus* ET34  
917 pediocin purification according to the following sequential steps: a) hydrophobic  
918 interaction, b) ion exchange, c) C18 SPE and d) reversed phase HPLC. Pediocin was  
919 indirectly quantified by the antimicrobial activity against *L. innocua* DPC3572 of each  
920 of the obtained fractions (S: cell free supernatant; FT: “flow through” sample that did  
921 not interact with the column; 30E: washing with 30% ethanol; 70IPA: elution with 70%  
922 isopropanol and 0.1% TFA; 150 mM: washing with 150 mM NaCl; 1M: elution with 1  
923 M NaCl. d) The mass spectrum corresponds to the lyophilized mixture of the different  
924 fractions eluted in HPLC after salts removal using an increasing gradient of acetonitrile.

925

926 **Figure 4.** Pareto chart and response surface plot illustrating the influence of temperature  
927 and HSB percentage in the medium on the antimicrobial activity of cell-free supernatant  
928 after 14 h of cultivation. The magnitude of each effect is represented in the Pareto chart  
929 by a column and a line crossing the columns that indicate how large an effect must be to  
930 be considered statistically significant. The effect estimates divided by their standard  
931 errors are sorted from the largest absolute value to the smallest one. The vertical line  
932 corresponds to a *p*-value of 0.1, which implies a 90% level of significance.

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934 **Figure 5.** Results of *Pediococcus pentosaceus* ET34 fermentation on LAPT medium  
935 supplemented with 20.6% HSB in 3-L Erlenmeyer flask at 36.8 °C. a) Cell-free  
936 supernatant antimicrobial activity (AU·mL<sup>-1</sup>) against *Listeria innocua* CLIST 2711 (red  
937 columns) and lactic acid (yellow line) and acetic acid (green line) concentrations (g·L<sup>-1</sup>)  
938 along the cultivation time. b) Arabinoxylan, xylose, cello-oligosaccharide, arabinose,  
939 glucose, formic acid, HMF and furfural concentrations (g·L<sup>-1</sup>) at the start and after 2, 4,  
940 6, 8, 10, 12 14 and 24 h.

941

942 **Figure 6.** Schematic representation of the planned integrated process for pediocin PA-1  
943 production and purification, and recycling of the main solvents. The isolation of the  
944 phenolic compounds from each aqueous phase and reuse of the respective phase-  
945 forming components are also illustrated.