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Selective carboxylate production from detoxified lignocellulosic prehydrolysates through bioaugmented anaerobic fermentation

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Lignocellulosic residues often require pretreatments to modify their polymeric structure and facilitate subsequent upcycling. Steam explosion, a common pretreatment method, generates microbial inhibitors like furfural and 5-hydroxymethylfurfural, making detoxification essential to mitigate their adverse effects. This study investigated the novel application of anaerobic fermentation (AF) for converting prehydrolysates from steam-exploded municipal green residues (MGR) into carboxylates and assessed the effect of activated carbon (AC) as a detoxifying agent to promote lignocellulose valorization. Bioaugmentation of the AF was applied for the selective production of lactic and acetic acids. Continuous stirred-tank reactors were fed with detoxified prehydrolysates under identical conditions (15-days of hydraulic retention time (HRT), 1 g COD/Ld, pH 4.5), with one of them bioaugmented with 10% (v/v) *Lactobacillus pentosus* MAX2. Due to the heterolactic metabolism of this bacterium, the bioaugmented reactor selectively produced 56.8% w/w lactic acid and 40.5% w/w acetic acid, with a total bioconversion of 45.2%. The presence of *L. pentosus* MAX2 in the microbial community along the AF process was confirmed both by PCR and DNA sequencing. These results do not only establish AF as a viable route for the valorization of lignocellulosic hydrolysates into high value commodity chemicals, but also demonstrate that bioaugmentation can effectively steer metabolic selectivity in mixed cultures, even under conditions where washout could pose a challenge.

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1. Introduction

Municipal green residues (MGR), including grass clippings, hedge trimmings, tree cuttings with bark, and leaves, are generated in substantial quantities in urban areas. Across the European Union, garden and park waste represents a considerable share of the approximately 229 million tonnes of municipal solid waste produced annually.[1] Despite their high availability, these residues are still primarily managed through conventional practices such as composting and landfilling.[2] The valorization of these residues is hindered by their lignocellulosic composition, consisting of cellulose, hemicellulose, and lignin. The heterogenous and complex structure of these components limits the biodegradability and bioconversion efficiency of lignocellulose, making pretreatments a necessary step to disrupt its structure. Among the available pretreatment methods, steam explosion is widely applied and generates a solid fraction retaining most of the cellulose and lignin and a liquid fraction rich in hemicellulosic sugars along with potential microbial inhibitors.[3,4]

While the negative impact of the inhibitors on bioethanol production from lignocellulosic sugars has been extensively studied, the potential advantages of employing mixed microbial cultures to mitigate inhibitor effects and valorize the entire organic fraction, not only sugars, remain insufficiently explored.[5] In the same line, extensive research has been performed on detoxifying lignocellulosic hydrolysates for bioethanol production.[6–9] However, little is known about the detoxification of feedstocks for anaerobic processes, whereby different microorganisms might respond differently.

Anaerobic fermentation (AF) is an innovative strategy for the valorization of lignocellulosic streams into high-value compounds that relies on mixed cultures. AF can be directed towards the accumulation of carboxylic acids (*i.e.*, citric (HCit), lactic (HLac), formic (HFor), acetic (HAc), propionic (HPro), isobutyric (isoHBut), butyric (HBut), iso-valeric (isoHVal), valeric (HVal), iso-hexanoic (isoHHex) and hexanoic acids (HHex)) by controlling operational conditions to inhibit methanogenic archaea. Among these acids, HAc is widely used industrially to produce vinyl acetate, a key precursor in vinyl plastic, latex paints, adhesives and textiles.[10] Likewise, HLac serves as

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a platform chemical with broad applications across the food, pharmaceutical, agricultural, and chemical sectors due to its role as a biodegradable monomer in the synthesis of polylactic acid.[11,12] Given the rapidly growing global demand for HLac, sustainable production routes based on AF of lignocellulosic residues represent a promising alternative to traditional fermentations relying on pure substrates and single strain cultivation. Because AF is conventionally carried out using anaerobic inocula sourced from anaerobic digesters, the resulting microbiome exhibits a wide range of microbial activities, often leading to effluents rich in a mixture of carboxylates.[13] This can be considered a problem, as acids extraction from the cultivation broth and purification may compromise the cost-efficient use of the carboxylates.

Bioaugmentation strategies in AF (*i.e.*, deliberate inoculation with a specific microorganism) offer a promising approach to steer metabolic selectivity and reduce the diversity of carboxylates produced in mixed-culture fermentations. By introducing microorganisms with defined metabolic pathways, bioaugmentation can selectively enrich specific carboxylates, thereby facilitating product recovery and purification. Furthermore, these strategies have shown to stabilize microbial communities and enhance process robustness under challenging environmental conditions,[14,15] providing a pathway toward viable valorization of lignocellulosic residues into platform chemicals.

While bioaugmentation with lactic acid bacteria has been investigated in AF,[16,17] its application to inhibitor-containing lignocellulosic prehydrolysates in continuous mixed-culture systems remains largely unexplored. The present work addresses these gaps by investigating the AF of lignocellulosic prehydrolysates derived from steam-exploded MGR using non-sterile mixed cultures in continuous stirred-tank reactors (CSTRs). To facilitate the conversion of the lignocellulosic stream, an activated carbon (AC) detoxification step was optimized. Aiming at the selective co-production of HLac and HAc as the principal carboxylates, this study compared a non-bioaugmented process with a bioaugmented counterpart supplemented with *Lactobacillus pentosus* MAX2, currently known as *Lactiplantibacillus pentosus*.[18] Process performance was evaluated in terms of carboxylic acid profiles, while microbial community was analyzed by PCR and DNA sequencing to link process outcomes with the microorganisms driving AF.

2. Materials and methods

2.1. Feedstock and inoculum sources

MGR were collected from the Migas Calientes composting facility (Madrid, Spain) and milled prior to storage. Steam explosion pretreatment was performed in a 2-L reactor under varying conditions (200–220 °C, 5–10 min) with different impregnation strategies, including water or H_2SO_4. The resulting slurries were separated by vacuum filtration through a 60 μm nylon membrane into a solid fraction and a liquid fraction (also called prehydrolysate). Prehydrolysates from all pretreatment conditions were combined to produce a uniform and representative MGR prehydrolysate for subsequent AF. Mesophilic anaerobic sludge from the Arroyo del Soto wastewater treatment plant (Móstoles, Comunidad de Madrid, Spain) was used as the inoculum for AF.

2.2. Experimental design and operating conditions

AF was performed in 1.5-L CSTRs with a working volume of 0.5 L with: (i) non-detoxified MGR prehydrolysate, (ii) detoxified MGR prehydrolysate, and (iii) detoxified MGR prehydrolysate in a bioaugmented system. In the last case, *L. pentosus* MAX2 (10% v/v, preculture $OD_{600nm} = 5.05$) was co-inoculated with the anaerobic sludge only at the start of the AF (no further addition of this bacterium was conducted at any time of the experimental time evaluated). *L. pentosus* MAX2 was selected on the basis of its xylose-fermenting capacity, developed through adaptive laboratory evolution in repeated anaerobic batch cultures under increasing xylose concentrations at acidic pH,[19] and the inherent tolerance of heterofermentative lactic acid bacteria to furanic inhibitors, including the partial bioconversion of 5-hydroxymethylfurfural (HMF) and furfural into less toxic derivatives.[20] Reactor performance was evaluated under steady-state conditions, which were achieved after a minimum of three HRTs and verified by consistent metabolite concentrations across multiple sampling points, providing internal analytical reproducibility.

AF was performed at 35 °C and temperature was maintained using a refrigerated/heating water circulator (CORIO CD-200F, Julabo GmbH, Germany). Process pH was daily monitored with a multi-parameter analyzer (Consort C3040, Belgium) and adjusted at 4.5 by adding NaOH (1.6 M) or HCl (3 M) after feeding. Reactors were operated at an organic loading rate (OLR) of 1 g COD/Ld and a hydraulic retention time (HRT) of 15 d, and were run for at least 3 HRTs or until steady-state conditions were achieved.

2.3. Detoxification treatment of feedstock

AC was added as detoxification agent to the prehydrolysates at concentrations of 10 g L^{-1} or 50 g L^{-1} and incubated for 4, 16, and 24 h to evaluate the removal inhibitor, namely HMF, furfural, 4-hydroxybenzaldehyde, and vanillin, as well as total phenols reduction. The range of AC concentrations (10–50 g L^{-1}) was selected based on reported effective detoxification of lignocellulosic hydrolysates within this interval.[21,22] These concentrations allow the evaluation of both moderate and intensive detoxification conditions. Contact times between 4 h and 24 h were selected based on previous studies demonstrating effective removal of furan derivates and phenolic compounds within this range.[23,24] Extended contact times up to 24 h were included to ensure adsorption equilibrium, as the removal of phenols and furans by activated carbon is governed by time-dependent adsorption kinetics, where slower intra-particle diffusion processes require longer incubation periods. After detoxification at optimized conditions (50 g AC/L and 16 h), the prehydrolysate was centrifuged for 30 min at 470 g at room temperature in a Thermo Scientific™ Heraeus Megafuge™ 16R centrifuge with Auto-Lock™ rotor system. The supernatant was filtered by using a pore size of 0.22 μm with

a bottle-top vacuum filter system and analyzed as described in Section 2.4 to assess inhibitors reduction. The resulting detoxified prehydrolysate was used as a feedstock for both non-bioaugmented and bioaugmented AF processes.

2.4. Chemical analytical methods

Total phenolic compounds concentration in the feedstock was determined using a modified Folin–Ciocalteu assay.[25] HMF, furfural, 4-hydroxybenzaldehyde, and vanillin, were determined by high-performance liquid chromatography (HPLC).[26]

Total solids (TS), volatile solids (VS) and ash in detoxified, non-detoxified MGR prehydrolysates and reactor effluents were measured according to standard protocols.[27] Total and soluble chemical oxygen demand (TCOD and SCOD, respectively) were analyzed with COD Cell Test Spectroquant® and measured on a Spectroquant® Prove 100 spectrophotometer (Merck, Germany). In the case of the detoxified prehydrolysate, TCOD and SCOD were identical, as the treatment included filtration through a 0.22 μm membrane (Materials & methods 2.3). Total Kjeldahl Nitrogen (TKN) was measured using standard protocols,[27] estimating the protein content by multiplying TKN by a conversion factor of 6.21. Ammonium (NH_4^+-N) concentration was determined using a photometric test kit (Spectroquant® Ammonium Cell Test, Supelco/Merck, Germany), according to the manufacturer's instructions. Total carbohydrates content was quantified using the phenol–sulfuric acid method,[28] and the carbohydrate composition was analyzed by HPLC equipped with an ion exchange column (CarboSep CHO-682 Pb, 7.8 × 300 mm, Concise) in an oven at 80 °C, using Milli-Q water as mobile phase and a refractive index detector (RID) working at 35 °C.

Short-chain fatty acids (SCFAs), ethanol (EtOH), citric acid (HCit), HFor and HLac were quantified by HPLC (1260 Agilent, USA) equipped with a RID, a Cation H Refill Cartridge Micro-guard pre-column (Bio-Rad), and an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm, Bio-Rad). Before injection, samples were centrifuged and filtered through 0.22 μm filters. A 20 μL injection volume was used, with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}. The oven and detector temperatures were set to 44 °C, and the RID was operated at 35 °C.

The bioconversion of the feedstock into recovered metabolites in the effluent was calculated as shown in eqn (1), where $COD_{metabolites,effluent}$ represents the sum of COD equivalents concentrations (g COD/L) for HCit, HLac, HFor, HAc, HPro, isoHBut, HBut, isoHVal, HVal, isoHex acid, HHex and EtOH. The $tCOD_{influent}$ refers to the total COD (g COD/L) of the feedstock employed to feed the reactors.

$$\text{Bioconversion}(\%) = \frac{\text{COD}_{\text{metabolites, effluent}}}{\text{tCOD}_{\text{influent}}} \times 100 \qquad (1)$$

2.5. Microbial community analysis

Samples from the initial inoculum as well as from the reactors after 14, 35 and 56 days were collected and stored at −20 °C. Microbial DNA was extracted from these samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, LCC), according to the supplier's instructions. The concentration and purity of the obtained DNA were evaluated by measuring the absorbance at 260, 280 and 230 nm with a microplate spectrophotometer (Omega Spectrostar BMC Labtech). The V3–V4 hypervariable regions of the 16S rRNA genes in the last DNA sample were amplified and sequenced by FISABIO (Valencia, Spain) to study the composition of bacterial and archaeal communities using an Illumina MiSeq platform.

A previously described, PCR-based method was used to detect the presence of *L. pentosus* in all DNA samples from both the bioaugmented and non-bioaugmented reactors.[29] To this end, a unique gene to *L. pentosus*, referred to as gyrase-Hsp90-histidin kinase-MutL (GHKL) domain-containing gene (GenBank: CP032757.1) was amplified by PCR using the extracted DNA, the standard conditions for Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the primers Pentosus-F (GCGGTATCGATTCGATTGGT) and Pentosus-R (TGATGTCAATCGCCTCTTGG). The amplicon with the expected size was extracted from an agarose gel (QIAquick PCR & Gel Purification Kit, QIAGEN GMBH) and analyzed by Sanger sequencing at Eurofins Genomics (Cologne, Germany).

3. Results and discussion

3.1. Detoxification

AF with the non-detoxified prehydrolysate resulted in total process inhibition and only those compounds supplied by the lignocellulosic prehydrolysate were detected in the effluent. Under these conditions, the microbial community was washed out and inhibitory compounds accumulated in the reactor (data not shown). Thus, a detoxification step was conducted prior using the prehydrolysates as feedstock for AF.

Under the evaluated detoxification conditions, inhibitor removal percentage increased concomitantly with the tested AC concentration. Detoxification treatment carried out for different time periods (4, 16, and 24 h) did not result in any significant differences in the concentration of HMF, furfural, 4-hydroxybenzaldehyde, vanillin, coumaric acid, and ferulic acid concentrations (Table 1). In contrast, significant differences in total phenols were observed between the 4 h and 16 h treatments, while extending the treatment to 24 h did not lead to any further phenol reduction (Table 1). Based on this, the addition of 50 g L^{-1} AC combined with a mixing time of 16 h was identified as the optimal detoxification condition for treating MGR

Table 1 Inhibitors reduction percentage (%) at different AC detoxification conditions

AC concentration (g L^{-1})	HMF, furfural, 4-hydroxybenzaldehyde and vanillin			Total phenols		
	4 h	16 h	24 h	4 h	16 h	24 h
10	46	44	45	26	44	45
50	98	98	98	58	76	76

prehydrolysates used as feedstock for AF in both bioaugmented and non-bioaugmented reactors.

As shown in Table 2, the detoxification of the MGR prehydrolysate led to notable changes in its composition. In particular, TCOD decreased by 21.4%, from 103.65 g L^{-1} in the non-detoxified sample to 81.50 g L^{-1} after treatment. This reduction was primarily driven by the effective removal of inhibitory compounds, including HMF, furfural, 4-hydroxybenzaldehyde, and vanillin, which decreased by 87.8, 89.6, 100, and 100%, respectively, together with a 71.4% reduction in total phenolic content. A minor contribution to the TCOD decrease arose from the partial adsorption of fermentable sugars onto the AC, as glucose, xylose, galactose, arabinose, and mannose were reduced by less than 10% following treatment. Based on standard theoretical oxygen demand (ThOD) conversion factors for hexoses and pentoses,[30] the COD losses attributable to sugars accounted for approximately 22% of the total TCOD reduction (~4.9 g COD per L out of 22.15 g COD per L), indicating that substrate availability for AF was not substantially compromised.

The selective action of AC toward inhibitory compounds over fermentable sugars is governed by differences in their physicochemical properties. Aromatic and conjugated inhibitors such as furfural, HMF, and phenolic compounds are preferentially adsorbed onto the hydrophobic, graphene-like surface of AC through π–π interactions and hydrophobic partitioning,[31] whereas polar sugars lack aromatic structures and are therefore only weakly adsorbed *via* hydrogen bonding.[32] This inherent selectivity explains the high removal of furanic and phenolic compounds (87–100%) alongside the minimal loss of fermentable sugars (<10%) observed under the optimized detoxification conditions, consistent with previous reports.[32,33]

The high efficiency removal of inhibitors with AC was in line with available literature. According to literature, optimized AC detoxification of acid-pretreated sorgum leaf waste removed up to 98% of furfural, 88% of HMF and significantly reduced HAc concentrations while preserving fermentable sugars, alongside further reduction in phenolic compounds by 55%, HMF by 64% and furfural by 41% in olive tree pruning hydrolysates after AC detoxification.[9,24] This selective removal of inhibitory compounds improved microbial growth and bioethanol production. The differential response of inhibitor removal to contact time reflected differences in adsorption kinetics across compound classes. Furanic compounds and simple aromatic aldehydes, as low-molecular-weight and highly diffusive species, rapidly reached adsorption equilibrium within 4 h, and extending the contact time did not result in further removal. In contrast, total phenols comprise a heterogeneous mixture, including higher molecular weight lignin-derived polyphenolics, which exhibit slower intraparticle diffusion within the AC structure.[32,33] As a result, longer contact times were required to approach equilibrium, explaining the increase in total phenol removal between 4 and 16 h and the absence of further improvement at 24 h. The dependence of removal efficiency on AC concentration reflected adsorption capacity limitations. At 10 g L^{-1}, the available adsorption surface was insufficient relative to the inhibitor load, leading to partial removal, whereas at 50 g L^{-1}, the higher number of available adsorption sites enabled near-complete removal of furanic compounds and aromatic aldehydes. Overall, the literature consistently demonstrates the effectiveness of AC in removing pretreatment-derived lignocellulosic inhibitors. However, studies applying detoxified prehydrolysates in mixed-cultures systems remain scarce and none of the existing reports address its application in AF.

The use of AC at 50 g L^{-1} represents a significant material input, making its cost and regeneration capacity important considerations for process scale-up. Previous studies have shown that spent AC from lignocellulosic hydrolysate detoxification can be regenerated through chemical treatment, reducing operational costs by up to 38% through successive reuse cycles.[34] In addition, the production of AC from lignocellulosic residues, including the solid fraction generated during steam explosion of MGR, offers a promising route for integrated biorefinery valorization and cost reduction.[24] A comprehensive techno-economic assessment of the integrated detoxification–bioaugmentation process, including AC regeneration strategies, remains important for future work.

Table 2 Composition of the non-detoxified and detoxified MGR prehydrolysate after AC treatment (50 g L^{-1} AC mixed for 16 h)[a]

	Lignocellulosic prehydrolysate	
	Non-detoxified (mean)	Detoxified (mean)
TCOD (g L^{-1})	103.65 ± 2.55	81.50 ± 0.30
SCOD (g L^{-1})	99.85 ± 1.35	—
TS (g L^{-1})	74.74 ± 0.31	64.74 ± 0.25
VS (g L^{-1})	69.22 ± 0.44	56.40 ± 0.10
NH_4^+-N (mg L^{-1})	83.11 ± 3.37	83.57 ± 2.59
N (mg L^{-1})	653.56 ± 13.94	285.02 ± 4.66
Proteins (mg L^{-1})	4.06 ± 0.09	1.77 ± <0.10
Glucose (g L^{-1})	15.86 ± <0.10	14.41 ± <0.10
Xylose (g L^{-1})	27.92 ± <0.10	25.48 ± <0.10
Galactose (g L^{-1})	4.49 ± <0.10	4.28 ± <0.10
Arabinose (g L^{-1})	2.73 ± <0.10	2.66 ± <0.10
Mannose (g L^{-1})	3.79 ± <0.10	3.51 ± <0.10
Citric acid (g L^{-1})	0.82 ± <0.10	0.58 ± <0.10
Lactic acid (g L^{-1})	0.75 ± <0.10	0.08 ± <0.10
Formic acid (g L^{-1})	1.61 ± <0.10	1.45 ± <0.10
Acetic acid (g L^{-1})	10.21 ± <0.10	9.62 ± <0.10
Propionic acid (g L^{-1})	0.04 ± <0.10	0.03 ± <0.10
HMF (g L^{-1})	1.31 ± <0.10	0.16 ± <0.10
Furfural (g L^{-1})	2.40 ± <0.10	0.25 ± <0.10
4-Benzaldehyde (g L^{-1})	0.07 ± <0.01	n.d
Vanillin (g L^{-1})	0.04 ± <0.01	n.d

[a] n.d. not detected.

3.2. Carboxylate production in bioaugmented and non-bioaugmented anaerobic fermentation of detoxified prehydrolysates

High selectivity in fermentation processes is often directed towards a single target compound, such as HLac. However, this approach typically relies on pure cultures or genetically modified microorganisms to enhance product yields, which

increases operational costs, process complexity and environmental burdens.[35,36] In contrast, the present study focused on the selective co-production of two carboxylates using mixed cultures.

Fig. 1 shows the metabolites detected at the steady state (after 56 days of process) during bioaugmented and non-bioaugmented AF of detoxified MGR prehydrolysates. Without bioaugmentation, carboxylate production at the steady state was clearly dominated by HBut, reaching 3.6 g L^{-1}. Lower concentrations of HAc and HLac (0.8 and 0.4 g L^{-1}, respectively) were also present, together with trace amounts of other minor products, such as HCit. This metabolite distribution is consistent with the typical metabolic behavior of mixed-culture AF, in which a diverse microbial community preferentially channels carbon toward different SCFAs, particularly HBut.[37]

In the reactor where the inoculum was bioaugmented, HLac became the predominant metabolite, reaching a concentration of 3.5 g L^{-1}, while HAc was the second most abundant acid (2.7 g L^{-1}). The detoxified MGR prehydrolysate contained 9.62 g L^{-1} of HAc (Table 2), which, was reduced to approximately 1.8 g L^{-1} after the 5.4-fold dilution to get desired OLR (1 g COD/L·d). In the non-bioaugmented reactor, the steady-state HAc concentration (0.8 g L^{-1}) was lower than this value, indicating net consumption by the microbial community. In contrast, the bioaugmented reactor reached 2.7 g per L HAc at steady state, exceeding the influent contribution and thus confirming net biological production, which was congruent with the heterolactic metabolism of *L. pentosus* MAX2. HCit, HFor and EtOH were detected at negligible concentrations in both reactors, with values lower than 0.3 g L^{-1} in all cases. This metabolic profile demonstrated that bioaugmentation successfully shifted the fermentation toward HLac and HAc production, creating a metabolic environment consistent with the physiological traits of *L. pentosus* MAX2. This strain is a facultative heterolactic HLac bacterium capable of metabolizing both hexoses and pentoses *via* the Embden-Meyerhof-Parnas and the phosphoketolase pathways, respectively. Its ability to produce HLac, HAc and other minor metabolites has been widely documented and linked with its ecological competitiveness,[38] supporting its suitability for bioaugmentation applications.

The marked differences in metabolite distribution between both reactors highlighted the strong impact of bioaugmentation on mixed-culture conducting AF. Low pH (4.5) maintenance in the system alone proved insufficient to achieve selective HLac production. The non-bioaugmented reactor remained dominated by HBut, suggesting that butyrate-producing *Clostridium* species can tolerate acidic conditions and remain metabolically competitive at pH 4.5. In contrast, *L. pentosus* MAX2 addition resulted in a profile dominated by HLac and HAc, demonstrating that bioaugmentation provided an additional selective pressure to shape metabolite profile beyond pH control. This metabolic shift was also reflected in bioconversion efficiency.

The difference in bioconversion efficiency between reactors was largely attributable to the distinct COD contributions of the metabolites produced. Since bioconversion was expressed on a COD basis, the higher value obtained in the non-bioaugmented reactor reflected the dominance of HBut (1.82 g COD per g), which carries a higher theoretical oxygen demand than HLac and HAc (1.07 g COD per g each). Accordingly, bioaugmentation did not reduce carbon conversion rates *per se*, but rather redirected carbon fluxes toward a more selective metabolites profile. Under steady-state conditions, COD removal was negligible (<5%). Total metabolite acidification efficiency reached 72.03 ± 8.11% and 57.01 ± 10.32% in the non-bioaugmented and bioaugmented reactors, respectively, while SCFA acidification efficiency was 67.59 ± 5.47% and 24.23 ± 4.37%, confirming that influent COD was predominantly recovered as fermentation products.

Several studies have reported that pure cultures of *L. pentosus*, as well as co-culture systems incorporating this microorganism, can produce high quantities of HLac and other

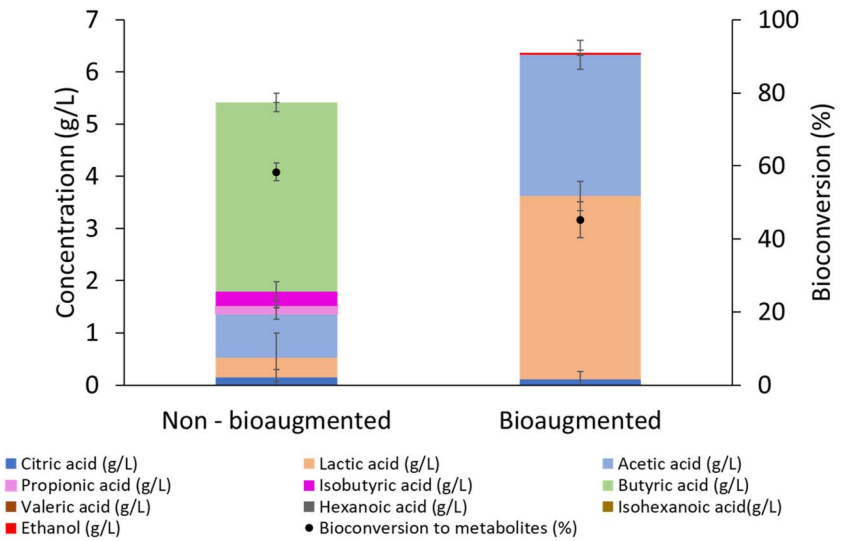

Fig. 1 Metabolites concentration (g L^{-1}) and bioconversion (%) at the steady state in both bioaugmented and non-bioaugmented reactors.

metabolites.[39,40] However, information on the effectiveness of bioaugmentation in mixed cultures for residue valorization toward selective production of acids remains limited. Expanding knowledge in this area as an alternative to pure cultures is particularly relevant because microbial diversity enhances process resilience to operational stresses, enables the degradation of the broad spectrum of organic compounds present in complex waste streams, and eliminates the need for sterile operating conditions.

High selectivity in fermentation processes is typically directed toward a single target compound, such as HLac, and is most commonly achieved using pure cultures or genetically modified microorganisms. While these systems can deliver high product titers and yields, they generally require strict sterile operation, and, in the case of genetically modified organisms, compliance with regulatory frameworks, all of which increase process complexity and cost. In contrast, the mixed-culture approach applied here prioritizes operational simplicity, process robustness, and the ability to valorize complex, non-sterile waste streams. Although carbon is inherently distributed across multiple metabolic pathways in such systems, resulting in lower selectivity compared to pure cultures, bioaugmentation enabled partial metabolic steering toward a targeted product spectrum. This was reflected in the co-production of HLac and HAc under non-sterile conditions using a lignocellulosic waste-derived substrate. These results supported the positioning of mixed-culture bioaugmented AF as a complementary approach to pure-culture fermentation, particularly in applications where feedstock complexity and process economics are key constraints.

3.3. Microbial community identification

3.3.1. Composition and diversity of microbial communities. The microbial composition in the bioaugmented and non-bioaugmented AF reactors was analyzed to evaluate the effect of *L. pentosus* MAX2 addition on the community structure. The same initial inoculum was used in both reactors and all operational parameters were maintained identical, except for the addition of *L. pentosus* MAX2. Therefore, any differences observed between the samples could be attributed to the influence of the bioaugmentation.

As shown in Fig. 2, the inoculum exhibited high biodiversity, a characteristic feature of microbiomes found in anaerobic digesters,[41] whereby those microbial systems are claimed to enhance resilience against environmental perturbations.[42,43] The major phyla present in the inoculum in relative abundance (%) were Chloroflexi (15.1%), Desulfobacterota (13.7%), Verrucomicrobiota (12.7%), and Planctomycetota (12.0%), among others. The relative abundance of microorganisms that could not be taxonomically identified accounted for approximately 5% of the total.

Comparison of the microbial community structure found in the inoculum and the reactors at steady state revealed a notable biodiversity reduction. This loss of diversity reflected a pronounced specialization of the microbiome in both reactors in response to the applied operational conditions. As a result of this selection process, Firmicutes became the predominant phylum in both steady-state reactors, accounting for 100% of the relative abundance (Fig. 2).

Members of the phylum Firmicutes are commonly found in AF systems and are recognized for their key roles in the hydrolytic and acidogenic stages of organic matter degradation.[44] During these phases, they degrade complex substrates and produce SCFAs.[45–47] The strong selection toward Firmicutes can be attributed to their notable physiological adaptations to acidic environments, such as the thick peptidoglycan layer in their cell wall,[47] which enables them to maintain metabolic activity under the controlled acidic pH conditions (pH = 4.5) applied in this study.

Operational conditions applied during AF imposed selective pressures that reduced the genus-level diversity (Fig. 3). In the non-bioaugmented AF, the microbial community was dominated by *Sporolactobacillus* (32.9%), *Clostridium* (28.8%) and *Lactobacillus* (23.6%), while *Ethanoligenens* (4.4%) and unidentified members of the Ethanoligenenaceae family (10.4%). By contrast, in the bioaugmented AF, only two genera were found: *Lactobacillus* (52.3%) and *Sporolactobacillus* (47.7%), both HLac bacteria. Consistent with literature reports, *Lactobacillus* has been shown to dominate AFs, reaching relative abundance of 83.4% when fermenting sterilized food waste slurry at pH 5, whereas lower relative abundance of 30.4% have been observed during fermentation of synthetic food waste at pH 4.5.[48,49]

As heterofermentative bacteria involved in the acidogenic phase of AF, *Lactobacillus* species are capable of producing HLac and other acids.[50] Moreover, their ability to tolerate acidic environments, such as the pH 4.5 used in this study, provides a critical selective advantage over acidic pH-sensitive microorganisms. *Sporolactobacillus*, which survives even under harsher conditions owing to its ability to form spores, exhibits significant biotechnological potential due to its capacity to metabolize various sugars into high-purity D-HLac through homofermentative pathways. Its optimal growth temperature is 35 °C,[51] thriving through the mesophilic conditions employed in this study. *Clostridium* species are capable of producing HBut, HAc, and other carboxylic acids alongside biohydrogen *via* diverse fermentative pathways, being commonly associated with the acidogenic phase of AF.[52] Similar to other dominant acidogenic bacteria, their resilience to fluctuating environmental conditions, supported by endospore formation and an optimal growth temperature between 35 °C and 37 °C, enables them to thrive under mesophilic conditions and selective pressures such as acidification.[53]

The combined effect of acidic pH tolerance, inhibitory metabolite production and efficient acid-generating metabolism explains the selective enrichment of *Lactobacillus* alongside *Sporolactobacillus* in the bioaugmented reactor (Fig. 3), ultimately facilitating the accumulation of HLac and HAc as principal fermentation products. The ecological success of these genera under acidic conditions is well-documented across diverse substrate types. These genera have been shown to become the predominant species in the microbial communities of various silages,[54,55] red sour soup AF,[56] and real food waste

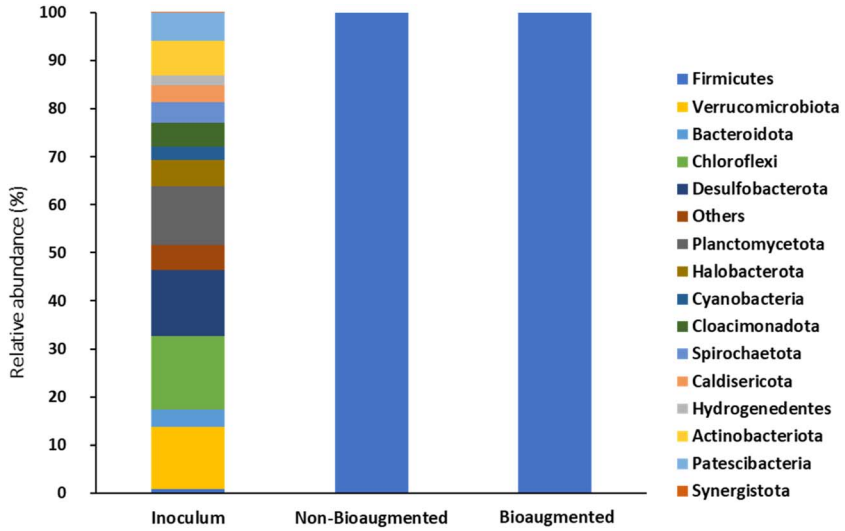

Fig. 2 Microbial community composition at the phylum level in the inoculum and steady-state samples of the non-bioaugmented and bioaugmented reactors.

AF,[57] demonstrating their robust adaptability to acidogenic environments regardless of feedstock composition.

The suppression of *Clostridium, Ethanoligenens* and Ethanoligenenaceae from the bioaugmented reactor reflects competitive inhibition driven by metabolic by-products produced by *L. pentosus* MAX2 and the enriched lactic acid bacteria community. When introduced into fermentation systems, bioaugmented microorganisms can exert competitive pressure on native microbial populations by producing metabolic by-products such as organic acids and EtOH that inhibit the growth of other microbes.[58] This competitive mechanism has been characterized in other bioaugmented fermentation systems in literature. For example, bioaugmentation of Baiju fermentation with the yeast *Wickerhamomyces anomalus* YM001 altered microbial community dynamics, which led to increased concentrations of ethyl acetate, ethyl decanoate and phenyl acetate, that suppressed native microorganisms' activity.[59] Similarly, reduced microbial diversity was observed after bioaugmentation in persimmon vinegar fermentation with *W. anomalus* ZX-1, *Lactobacillus plantarum* CGMCC 24035 and *Lactobacillus acidophilus* R2, further illustrating how introduced strains can restructure microbial communities.[60] In the present study, the high HLac and HAc production by *L. pentosus* MAX2, along with the low pH, generated strongly acidic conditions that selectively inhibited acid-sensitive genera, such as *Clostridium* (the main HBut producer), while favoring acid-tolerant HLac bacteria. This competitive pressure steered the fermentation toward the desired selective production of HLac and HAc, demonstrating that bioaugmentation can simultaneously drive microbial community restructuring and metabolic selectivity.

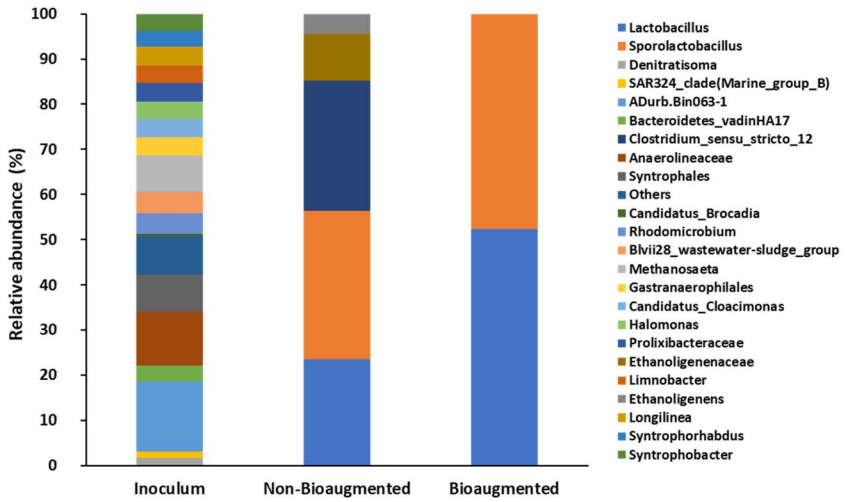

Fig. 3 Microbial community composition at the genus level in the inoculum and at the steady-state of the non-bioaugmented and bioaugmented reactors.

While this selective enrichment supports the targeted metabolic performance, it also has important ecological implications. The reduction in microbial diversity observed in the bioaugmented reactor, from five dominant genera to two, entailed both functional advantages and potential long-term limitations. While the simplified community enabled a stable and selective product profile under the tested conditions, the associated decrease in functional redundancy may reduce resilience to environmental perturbations compared to the more diverse non-bioaugmented system. In particular, the absence of *Clostridium* may limit the capacity of the community to adapt to variations in feedstock composition. At the same time, the persistence of *Sporolactobacillus*, a spore-forming genus, provided a degree of intrinsic resilience given its ability to withstand adverse conditions. Within the scope of this study, the functional capacity of the simplified community was proved sufficient to sustain selective HLac and HAc production over the experimental period.

At this point, it should be also highlighted that the microbial community analysis was conducted using 16S rRNA amplicon sequencing. As a result, the relationship between dominant taxa and the observed metabolite profiles was inferred from well-established metabolic traits of the identified genera offering valuable insights.

3.3.2. Identification and persistence of *L. pentosus* MAX2. To assess the presence of *L. pentosus* in the bioaugmented bioreactor, a GHKL domain-containing gene (named LP GHKL in Fig. 4), which is unique to this specie, was targeted using a species-specific primer pair.[29] The GHKL domain-containing gene encodes a protein belonging to the GHKL ATPase superfamily (Gyrase, Hsp90, Histidine Kinase, and MutL), which is involved in fundamental prokaryotic processes such as signal transduction and DNA repair. Its use as a species-specific marker for *L. pentosus* is not associated with a unique metabolic function, but rather with its sequence divergence from homologous genes in closely related species. Comparative genomic analysis of 180 *Lactobacillus* genomes demonstrated that this gene provides sufficient resolution for species-level discrimination.[29] This level of resolution is required because *L. pentosus*, *L. plantarum*, and *L. paraplantarum* share 99.7–99.9% 16S rRNA gene sequence similarity, which prevents reliable differentiation using standard amplicon sequencing. This approach was more convenient than targeting the V1–V3 variable region of the 16S rRNA gene because: (i) several *Lactobacillus* species share highly similar 16S rRNA gene sequences,[29] (ii) discrimination between closely related genera can be challenging when short hypervariable regions are sequenced instead of the full-lenght 16S rRNA gene,[61] and (iii) the *L. pentosus*-specific primer pair enables accurate identification of this specie in mixed cultures.

Fig. 4 shows that only the samples from bioaugmented AF yielded a band with the expected size (146 bp) corresponding to

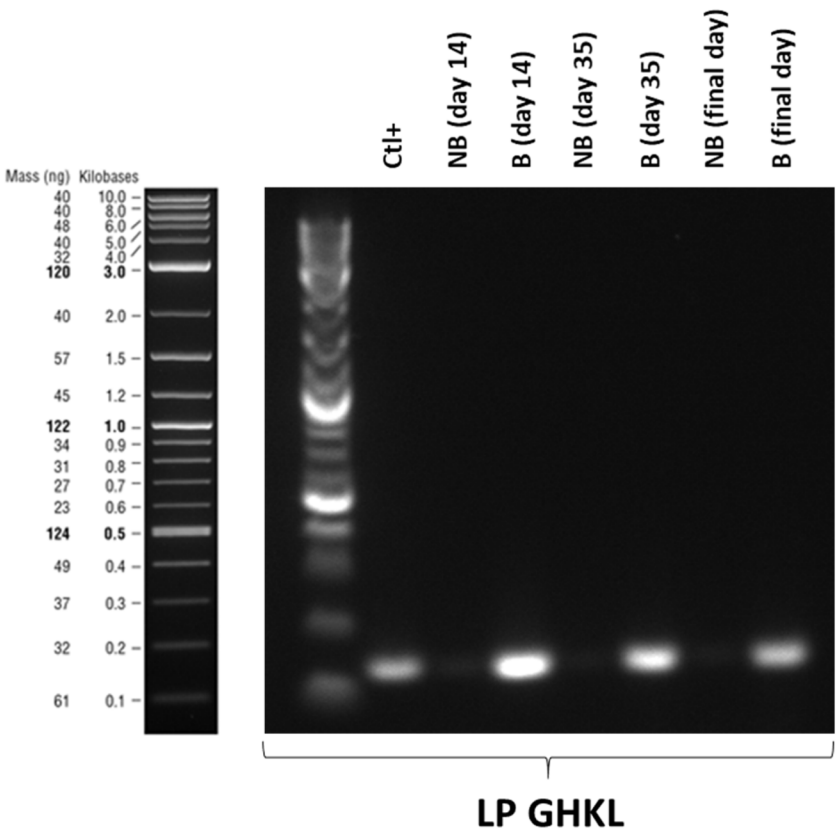

Fig. 4 Analysis of *L. pentosus* presence in non-bioaugmented (NB) and bioaugmented (B) reactors over the course of AF (days 14, 35 and 56 are represented).

the GHKL domain-containing gene. This confirmed that the introduced *L. pentosus* MAX2, inoculated only once at the start of the process, was able to persist in the mixed culture until the end of the AF. This persistence is particularly noteworthy given that bioaugmentation in continuous anaerobic systems is often challenged by the washout of introduced strains, especially under feeding conditions with relatively short HRTs.[62] The persistence of *L. pentosus* MAX2 in the bioaugmented reactor could be attributed to a combination of complementary factors. The predominance of xylose in the detoxified pregydrolysate may have provide a competitive advantage, consistent with its efficient pentose metabolism *via* the phosphoketolase pathway. Its reported tolerance to lignocellulosic inhibitors,[19,20] may have also contributed to its competitive advantage in the system. Furthermore, the rapid growth kinetics of *Lactobacillus* species, with doubling times typically ranging from 1 to 3 h under favorable conditions, are markedly shorter than the applied HRT of 15 d, enabling sustained population maintenance through active growth rather than passive retention. At the same time, the operating pH of 4.5 imposed a strong selective pressure that favored acid-tolerant lactic acid bacteria while limiting the competitiveness of other genera. This last advantage was reinforced by the prior adaptation of the MAX2 strain to acidic conditions,[19] which enhanced its fitness relative to both its parental strain and indigenous microbial populations. The persistent presence of *L. pentosus* MAX2 demonstrated its competitive fitness and successful integration into the microbial community, which is essential for achieving the targeted metabolic outcomes in bioaugmented systems. It is worth mentioning that using the GHKL domain-containing gene (complete or the expected PCR product) in a BLASTn against either the Core nucleotide BLAST or RefSeq Genome databases for *Lentilactobacillus parabuchneri*, another heterolactic bacteria, did not retrieve a sequence with significant similarity.

The same initial inoculum was used in both reactors, and all operational parameters were kept identical, with the sole exception of the addition of *L. pentosus* MAX2. The presence of *L. pentosus* MAX2 was consistently confirmed only in the bioaugmented reactor across all sampling points, and the extent of the community shifts was unlikely to be explained by stochastic variation alone. In addition, the observed community structures were consistent with the corresponding metabolite profiles in each reactor, supporting a mechanistic link between microbial composition and process performance. These findings position bioaugmentation as a promising strategy to overcome the inherent metabolic variability of mixed-culture AF, with direct implications for the design of selective and resilient bioprocesses.

4. Conclusions

This study demonstrated that combining substrate detoxification with bioaugmentation effectively enhanced AF performance to selectively produce HLac and HAc. Detoxification with AC removed key inhibitory compounds from the feedstock, while bioaugmentation with *L. pentosus* MAX2 promoted a stable and selective metabolic profile. Microbial community analyses revealed a strong selection towards Firmicutes. The non-bioaugmented reactor presented *Lactobacillus, Clostridium* and *Sporolactobacillus*, whereas the microbiome in the bioaugmented reactor was more specialized, dominated by *Lactobacillus* and *Sporolactobacillus*, and presented a greater reduction in microbial diversity. Molecular confirmation using a species-specific GHKL domain-containing gene verified that *L. pentosus* MAX2 was exclusively present in the bioaugmented reactor and persisted during the complete AF process. This study makes a substantial contribution to the development of sustainable bioprocesses for upcycling lignocellulosic residues containing microbial inhibitors.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data for this article are available at Zenodo at https://doi.org/10.5281/zenodo.20407474.

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