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Comparative LC-MS profiling and antimicrobial evaluation of Kefir-derived and *Lactobacillus*-derived postbiotics

Análisis comparativo mediante LC-MS y evaluación antimicrobiana de postbióticos derivados del kéfir y de *Lactobacillus*

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ABSTRACT

Kefir harbours a mixed community of lactic acid bacteria and yeasts, and the postbiotics obtained from it have attracted interest as natural functional ingredients with antimicrobial and health-promoting properties. It is not established, however, whether a postbiotic obtained directly from whole kefir grains differs from one produced by *Lactobacillus* strains isolated from the same grains. The objective of this study was therefore to compare the metabolite profile and the antimicrobial activity of a kefir-derived postbiotic (KP) and a *Lactobacillus*-derived postbiotic (LP). Cell-free preparations were obtained by controlled fermentation, centrifugation and membrane filtration. Their metabolite composition was characterized qualitatively by Liquid Chromatography–Mass Spectrometry (LC-MS), and their antimicrobial activity was determined by viable count reduction against Staphylococcus aureus ATCC43303 y Candida albicans ATCC10231 after 12 h of contact. Both preparations reduced the viable counts of both test organisms. KP reduced *S. aureus* by 2.99 ± 0.04 $\log_{10}$ CFU/mL and *C. albicans* by 1.74 ± 0.05 $\log_{10}$ CFU/mL, whereas LP reduced both organisms to below the detection limit of the plating method, corresponding to reductions of at least 3.02 ± 0.01 and 4.11 ± 0.01 $\log_{10}$ CFU/mL, respectively. The difference between the two preparations was statistically significant against *C. albicans* ($P < 0.001$) but not against *S. aureus* ($P = 0.28$), where both preparations approached the detection limit of the assay. The metabolite profiles of the two preparations largely overlapped: 18 of the annotated compounds formed a shared core that contained the principal antimicrobial organic and aromatic acids, while only a small number of features differentiated them, namely ascorbic acid and acetylated aromatic amino acids in KP, and free valine and short peptides in LP. Because the LC-MS characterization was qualitative and based on a single injection per preparation, and because no uninoculated-medium blank was analysed, these compositional differences should be regarded as preliminary. Within these limitations, the results suggest that the microbial source may influence both the metabolite profile of kefir postbiotics and, against *C. albicans*, their antimicrobial performance, and that both preparations merit further evaluation as natural antimicrobial agents for food applications.

Key words: Antimicrobial activity; Kefir; LC-MS; Lactobacillus; Postbiotics; Functional foods

RESUMEN

El kéfir alberga una comunidad mixta de bacterias lácticas y levaduras, y los postbióticos que se obtienen de él han despertado interés como ingredientes funcionales naturales con propiedades antimicrobianas y promotoras de la salud. No obstante, no está establecido si un postbiótico obtenido directamente de los granos enteros de kéfir difiere de uno producido por cepas de *Lactobacillus* aisladas de esos mismos granos. El objetivo de este estudio fue, por lo tanto, comparar el perfil de metabolitos y la actividad antimicrobiana de un postbiótico derivado del kéfir (KP) y de un postbiótico derivado de *Lactobacillus* (LP). Las preparaciones libres de células se obtuvieron mediante fermentación controlada, centrifugación y filtración por membrana. Su composición metabólica se caracterizó cualitativamente mediante Cromatografía Líquida–Espectrometría de Masas (LC-MS), y su actividad antimicrobiana se determinó por reducción del recuento viable frente a *Staphylococcus aureus* ATCC43303 y *Candida albicans* ATCC10231 tras 12 h de contacto. Ambas preparaciones redujeron los recuentos viables de ambos microorganismos. El KP redujo *S. aureus* en 2,99 ± 0,04 $\log_{10}$ UFC/mL y *C. albicans* en 1,74 ± 0,05 $\log_{10}$ UFC/mL, mientras que el LP redujo ambos microorganismos por debajo del límite de detección del método de recuento, lo que corresponde a reducciones de al menos 3,02 ± 0,01 y 4,11 ± 0,01 $\log_{10}$ UFC/mL, respectivamente. La diferencia entre ambas preparaciones fue estadísticamente significativa frente a *C. albicans* ($P < 0,001$), pero no frente a *S. aureus* ($P = 0,28$), donde ambas preparaciones se aproximaron al límite de detección del ensayo. Los perfiles metabólicos de ambas preparaciones se solaparon en gran medida: 18 de los compuestos identificados conformaron un núcleo común que contenía los principales ácidos orgánicos y aromáticos antimicrobianos, mientras que solo un número reducido de compuestos las diferenció, a saber, ácido ascórbico y aminoácidos aromáticos acetilados en el KP, y valina libre y péptidos cortos en el LP. Dado que la caracterización por LC-MS fue cualitativa y se basó en una única inyección por preparación, y que no se analizó un blanco de medio sin inocular, estas diferencias composicionales deben considerarse preliminares. Dentro de estas limitaciones, los resultados sugieren que la fuente microbiana puede influir en el perfil metabólico de los postbióticos de kéfir y, frente a *C. albicans*, en su desempeño antimicrobiano, y que ambas preparaciones merecen una evaluación adicional como agentes antimicrobianos naturales para aplicaciones alimentarias.

Palabras clave: Actividad antimicrobiana; Kéfir; LC-MS; Lactobacillus; Postbióticos; Alimentos funcionales.

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INTRODUCTION

The World Health Organization defines health as a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity [1], and diet influences each of these dimensions. Food components alter the composition and activity of the gut microbiota, and those changes are relayed to host physiology [2]. As the interplay between the microbiome, immune regulation and general health has been mapped in finer detail, attention has turned to food-derived molecules capable of acting on gut health, and postbiotics are now among the most heavily studied of them [3].

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines a postbiotic as a preparation of non-viable microorganisms, or of their constituents, that confers a health benefit on the host [4].

Probiotics must stay alive through processing and storage; postbiotics need not, which makes them safer to handle, more stable on the shelf, and easier to work into food systems on an industrial scale. What such a preparation actually contains varies, since its constituents arise partly from microbial metabolism and partly from the inactivated cells themselves. Short-chain fatty acids, organic acids, vitamins, enzymes, teichoic acids, antimicrobial peptides, exopolysaccharides and various cell-wall-associated fragments have all been reported [5, 6].

Postbiotics have been grouped both by what they are made of and by what they do in the body. Their biological activities span antimicrobial, antioxidant, anti-inflammatory, antihypertensive, antiproliferative, hypocholesterolemic and immunomodulatory effects [7].

This functional breadth explains the considerable research effort directed at incorporating postbiotics into foods, where they can extend shelf life, improve microbiological safety and open the way to new functional products [8].

Kefir is a traditional fermented milk drink, and the fermentation is carried out not by a single starter but by a mixed community of lactic acid bacteria, acetic acid bacteria and yeasts held together in kefiran, the polysaccharide matrix that gives the grains their structure. The grains themselves are more than a vehicle for that community: they also carry cell fragments, proteins and exopolysaccharides, any of which may contribute to the biological activity attributed to them [9, 10].

The current ISAPP definition goes further: inactivated or dried kefir grains qualify as postbiotic preparations in their own right [4, 9]. Antimicrobial and health-promoting effects have been documented for kefir and for preparations derived from it. What remains unsettled is how much of that activity belongs to postbiotics taken directly from whole grains and how much to those produced by individual bacterial isolates cultured from kefir [10, 11].

Postbiotics are chemically heterogeneous and complex, which makes their characterization difficult. A range of techniques has been applied to them, qualitatively and quantitatively, with the choice in any given case resting on what the study sets out to show and on the physicochemical behaviour of the metabolites being targeted [7, 12].

Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), Thin-Layer Chromatography (TLC) and spectroscopic methods all feature in the postbiotic literature. Liquid Chromatography coupled to Mass Spectrometry (LC-MS) has since taken over much of this ground. It is sensitive and selective, and it detects a wide range of the compounds generated during fermentation, which makes it well suited to profiling a metabolite pool comprehensively rather than one compound at a time [13].

Fermented dairy matrices have attracted sustained interest in food composition research, because fermentation generates a considerable variety of organic acids, peptides, amino acids and other bioactive metabolites. Kefir postbiotics, and those from the *Lactobacillus* strains that live in kefir, are correspondingly heterogeneous metabolic systems. Their chemical compositions have rarely been compared directly [14].

The antimicrobial properties of kefir-derived preparations have been examined previously, but rarely in the same study as their chemical composition. Work that pairs detailed LC-MS profiling with a head-to-head antimicrobial comparison of postbiotics from different kefir sources is scarce [13, 14].

The present study was designed to address this gap. Two postbiotic preparations were compared, one obtained directly from kefir grains and the other from Lactobacillus strains previously isolated from the same kefir, so that any difference between them could be examined in relation to their microbial origin.

The compositional signature of each preparation was determined by LC-MS, and their antimicrobial activity was assessed by viable count reduction assays against *Staphylococcus aureus* ATCC43303 and *Candida albicans* ATCC10231. Relating the chemical composition of the two preparations to their activity was intended to examine whether the microbial source influences the performance of a kefir postbiotic, and to indicate the potential of such preparations as natural bioprotective agents and functional food ingredients.

MATERIALS AND METHODS

Kefir grains

Kefir grains were purchased from a commercial local supplier (Mayasan-Biotech, Türkiye) and shipped to the laboratory under refrigeration. All subsequent procedures were carried out aseptically.

Isolation and identification of *Lactobacillus*

Lactobacillus strains were isolated following Kim *et al*. [15], with slight modifications. Kefir was prepared by inoculating 10 g of grains into 100 mL of pasteurized whole milk and holding the mixture at 25 °C for 48 hours (h). The fermented kefir was then streaked onto De Man, Rogosa and Sharpe agar (MRS agar, Merck, Darmstadt, Germany) and incubated anaerobically at 37 °C for 48 h, the plates being held in an anaerobic jar (116387, Merck, Darmstadt, Germany) placed inside an incubator (UN 55, Memmert GmbH + Co. KG, Schwabach, Germany). Deoxyribonucleic acid (DNA) was recovered from the isolates with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according

to the manufacturer's protocol, and identification rested on 16S ribosomal RNA (rRNA) sequencing with primers 27F and 1492R (Qiagen, Hilden, Germany) [16].

Preparation of kefir-derived postbiotic (KP)

Grains were activated as described by Garofalo *et al*. [17], with slight modifications: 10 g of grains went into 100 mL of pasteurized whole milk and were incubated at 25 ± 1 °C for 48 h, after which sterile gauze was used to separate the grains from the fermented milk. Three consecutive activation cycles were run in this way, so that the microbial population entered the experiment in a stable and active state [18].

Activated grains were transferred to MRS broth and incubated at 30 °C for 24 h. The cultures were then centrifuged at 20,000 × g for 20 min at 4 °C in a refrigerated centrifuge (5810 R, Eppendorf, Hamburg, Germany), and the recovered supernatants were passed through sterile 0.45 µm membrane filters (Isolab Laborgeräte GmbH, Eschau, Germany). This cell-free filtrate is the preparation referred to throughout as the kefir-derived postbiotic (KP) [19].

Preparation of *Lactobacillus*-derived postbiotic (LP)

Lactobacillus isolates recovered from kefir were grown individually in MRS broth at 37 °C for 24 h. Each culture was then subcultured once, a 10% (v/v) inoculum going into fresh broth under the same conditions, so that the cells were metabolically active when the preparation began [20].

The cultures were then centrifuged at 20,000 × g for 20 min at 4 °C, and the supernatants were clarified by 0.45 µm membrane filtration (Isolab Laborgeräte GmbH, Eschau, Germany) to remove any remaining bacterial cells. These cell-free preparations are the *Lactobacillus*-derived postbiotics (LP) [19].

Test microorganisms

Staphylococcus aureus ATCC43303 and *Candida albicans* ATCC10231 served as test organisms for both KP and LP. Both strains came from the culture collection of the Faculty of Veterinary Medicine, Sivas Cumhuriyet University.

C. albicans was cultivated in Sabouraud Dextrose Broth (SDB, Merck, Darmstadt, Germany) at 35 °C for 24–48 h, *S. aureus* in Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany) at 37 °C for 24 h. Suspensions were brought to the 0.5 McFarland standard (OD625 = 0.10), roughly 1.5×10^8 CFU/mL, and diluted down to a working inoculum of 105 CFU/mL. Each inoculum was verified by serial dilution and plating, Mannitol Salt Agar (MSA, Merck, Darmstadt, Germany) for *S. aureus*, incubated at 37 °C for 24 h, and Sabouraud Dextrose Agar (SDA, Merck, Darmstadt, Germany) for *C. albicans*, at 35 °C for 48 h. Counts are expressed as $\log_{10}$ CFU/mL.

Antimicrobial activity assay

Viable count reduction assays were used to measure the antimicrobial activity of KP and LP. One millilitre of pathogen suspension (approximately 10^5 CFU/mL) was combined aseptically with 1 mL of each postbiotic preparation, and the mixtures were incubated at 23 °C for 12 h. Serial decimal dilutions were then prepared in sterile physiological saline (0.9%) and plated on MSA for *S. aureus*, incubated at 37 °C for 24 h, and SDA for *C. albicans*, at 35 °C for 48 h. Survivors were enumerated as CFU/mL, with activity expressed as the $\log_{10}$ reduction against the initial count. All assays were performed in three independent experiments [21].

LC-MS analysis

The profiling protocol was adapted from Jeevan *et al*. [22]. Analyses were run on an Agilent 6530 Q-TOF LC-MS system (Agilent Technologies, Santa Clara, CA, USA), whose mass accuracy and low-abundance sensitivity suited the trace metabolites expected in these preparations. Compounds were separated on an InertSustain AQ-C18 column (3 µm, 4.6 × 150 mm; GL Sciences, Tokyo, Japan), aphase that holds the moderately polar constituents of fermented dairy well. The column was kept at 35 °C, the injection volume was 3 µL, and each run lasted 33 min.

The mobile phase paired 0.1% formic acid in water (A) with acetonitrile (B), delivered at 0.5 mL/min. Metabolites of differing polarity were resolved by gradient elution: 0–1 min, 5% B; 1–14 min, 20% B; 14–22 min, 40% B; 22–30 min, 90% B; and 30.01–33 min, 5% B to re-equilibrate the column.

Detection used an electrospray ionization (ESI) source in both positive and negative polarity. Drying gas temperature was 350 °C, nitrogen flow 11 L/min, nebulizer pressure 35 psi and capillary voltage 3500 V; the fragmentor was held at 150 V, and collision energies of 10, 20 and 40 eV were applied for MS/MS acquisition. Spectra were collected over m/z 30–1400, with continuous calibration on reference ions at m/z 121.0508 and 922.0098 in positive mode and m/z 112.9855 and 966.0007 in negative mode.

Chromatographic and spectral data were processed using Agilent MassHunter software (Agilent Technologies, Santa Clara, CA, USA). Peak detection was performed automatically and verified manually when necessary. For each detected compound, retention time, ionization mode, experimental mass, theoretical exact mass, and mass error were recorded. Compound identification was carried out at a putative level (MSI level 3) by matching the measured accurate masses (within 5 ppm) against the theoretical exact masses of candidate metabolites; isobaric and stereochemical isomers were not resolved. No absolute or relative quantification was performed; the comparison between KP and LP was therefore based solely on the presence or absence of annotated features.

A single injection of one cell-free preparation per group was analysed; the LC-MS comparison is therefore qualitative and was not designed to assess analytical reproducibility. Because accurate-mass annotation does not resolve isomers, isobaric and stereochemical isomers (e.g., L-isoleucine/L-leucine; D-/L-forms) were not differentiated, and no uninoculated-medium (MRS) blank was subtracted. The full annotation list (molecular formula, ion species, theoretical m/z, retention time and mass error for KP and LP) is provided in Supplementary TABLE S1.

Statistical analysis

All antimicrobial assays were performed in three independent experiments, and the results are expressed as mean ± standard deviation (SD) of the $\log_{10}$ CFU/mL values (n = 3). For each experiment the reduction was calculated as the difference between the initial and the final count. Counts falling below the detection limit of the plating method (<1.00 $\log_{10}$ CFU/mL) were treated as censored and assigned the value of the detection limit; the corresponding reductions are therefore conservative minimum estimates, so that any difference involving the *Lactobacillus*-derived preparation is underestimated rather than overstated. Because the substituted values carry almost no variance, however, the standard deviations reported for LP, and the test statistics that depend on them, should be regarded as approximate.

The effects of the test organism, of the postbiotic preparation and of their interaction on the $\log_{10}$ reduction were evaluated by two-way analysis of variance followed by Tukey's honestly significant difference test, and the reductions produced by KP and LP against each organism were additionally compared by Welch's t-test, which does not assume equal variances. Within each preparation the final count was compared with the initial count by a paired t-test. Homogeneity of variance across the four experimental groups was verified by Levene's test (P = 0.156); given the small number of replicates, normality was assumed rather than formally tested. A probability of $P < 0.05$ was accepted as statistically significant. All analyses were carried out using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

LC-MS profiling of kefir-derived and *Lactobacillus*-derived postbiotics

The aqueous fractions of the KP and LP cell-free supernatants were profiled on an Agilent 6530 Q-TOF instrument in both positive (ESI+) and negative (ESI−) modes. All annotations were obtained at the putative level (MSI level 3) by accurate-mass measurement within 5 ppm; the analysis was qualitative, and the two preparations were compared solely on the basis of the presence or absence of the annotated features. No uninoculated-medium (MRS) blank was analysed, and no replicate injections were performed, so the annotations reported below are putative and the comparison is provisional (see Limitations of the study).

Twenty-one features were annotated in KP and twenty-two in LP (TABLE I). The two preparations shared a common core of eighteen metabolites and differed in only a small number of features, which suggests that KP and LP may be compositional variants of the same kefir-derived metabolic background rather than chemically unrelated products. Because the analysis was qualitative, based on a single injection per preparation and performed without a medium blank, this interpretation is provisional.

TABLE I
Unique and shared metabolites identified in the kefir-derived postbiotic (KP) and the Lactobacillus-derived postbiotic (LP)

Detected only in KP (acidic / antioxidant character) — 3	Shared core — 18	Detected only in LP (proteolytic / peptide character) — 4
L-Ascorbic acid (vitamin C)	Spermidine	L-Valine
Acetyl-L-tyrosine	Lysine	L-Prolyl-L-leucine (dipeptide)
N-Acetyl-D-phenylalanine	L-Proline	L-Leucyl-L-valine (dipeptide)
	Guanine	Prolyl-valyl-leucine (tripeptide)
	L-Methionine	
	Hypoxanthine	
	Pyroglutamic acid	
	Xanthine	
	L-Isoleucine	
	L-Tyrosine	
	Succinic acid	
	L-Leucine	
	Phenylalanine	
	L-α-Hydroxyisovaleric acid	
	Tryptophan	
	Hydroxyphenyllactic acid	
	N-Acetyl-leucine	
	3-Phenyllactic acid	

Note: KP: kefir-derived postbiotic; LP: *Lactobacillus*-derived postbiotic; LC-MS: Liquid Chromatography–Mass Spectrometry; MSI: Metabolomics Standards Initiative. Annotations are putative (MSI level 3), based on accurate-mass matching within 5 ppm; isobaric and stereochemical isomers were not resolved and no quantification was performed. The principal antimicrobial acids (3-phenyllactic, hydroxyphenyllactic and succinic acids) and spermidine fall within the shared core and are therefore not unique to either preparation.

The shared core comprised free amino acids (proline, methionine, tyrosine, isoleucine, leucine, phenylalanine, tryptophan and lysine), the polyamine spermidine, the purine bases guanine, hypoxanthine and xanthine, and a group of organic and aromatic acids, succinic acid, L-α-hydroxyisovaleric acid, hydroxyphenyllactic acid and 3-phenyllactic acid, together with pyroglutamic acid and N-acetyl-leucine. The main antimicrobial organic and aromatic acids (succinic, hydroxyphenyllactic and 3-phenyllactic acids) fell within this shared fraction, so both postbiotics contained them. Spermidine was likewise detected in both samples and is consequently part of the shared core rather than a feature unique to LP.

KP was distinguished by three features not detected in LP: L-ascorbic acid and two acetylated aromatic amino acid derivatives (acetyl-L-tyrosine and N-acetyl-D-phenylalanine). LP was distinguished by four features absent from KP: free L-valine and three short peptides, the dipeptides L-prolyl-L-leucine and L-leucyl-L-valine and the tripeptide prolyl-valyl-leucine. The LP-specific pattern (a liberated branched-chain amino acid accompanied by di-/tripeptides) is consistent with a more proteolytically active preparation, whereas the KP-specific pattern (ascorbic acid and acetylated aromatic amino acids) may reflect KP's distinct production route, in which the grains were first activated in milk before transfer to MRS broth.

Each postbiotic was analysed in both ionization polarities, and the two total-ion chromatograms are presented together for each sample (FIG. 1: KP, ESI− upper and ESI+ lower panel; FIG. 2: LP, ESI− upper and ESI+ lower panel). Presenting both polarities allows the preparations to be compared within the same ionization mode, which is the appropriate basis for qualitative comparison; absolute intensities still differ between polarities, so the upper and lower panels of a given figure are not mutually quantitative.

In negative mode, both samples were dominated by early-eluting polar acids, amino acids and related metabolites, with the antimicrobial aromatic acids appearing later. The principal difference between the two negative-mode profiles was an intense, sharply resolved peak at ~3.3 min in KP that was absent from LP; this feature is putatively annotated as L-ascorbic acid,

consistent with the table-level finding that ascorbic acid was detected only in KP. Succinic acid (~5.5 min), hydroxyphenyllactic acid (~12.4 min) and 3-phenyllactic acid (~20.1 min) were present in the negative-mode traces of both samples, in line with their assignment to the shared core.

In positive mode, both samples showed a comparable cluster of polar species between ~2 and 6 min (spermidine, lysine, proline, methionine and the branched-chain amino acids) and were dominated by an intense peak at ~8.3 min, co-eluting with phenylalanine (annotated in negative mode at 8.26 min; Supplementary TABLE S1), followed by tryptophan at ~12 min. The features annotated as the LP-specific peptides (L-prolyl-L-leucine and L-leucyl-L-valine at ~10 min and prolyl-valyl-leucine at ~16 min) appeared only as minor positive-mode signals and represented the main qualitative difference between the two positive-mode profiles. This LP-specific enrichment in short peptides, together with the liberated branched-chain amino acid valine, is in line with untargeted LC-MS studies of kefir fermentation, which report a progressive increase in amino acids and peptides as microbial proteolysis advances [14].

Taken together, the total-ion chromatograms of the two preparations (FIG. 1 and FIG. 2) were broadly similar, consistent with the large shared core of annotated metabolites summarized in TABLE I; the differences between them lay mainly in a small number of features rather than in the overall profile. Because the analysis was qualitative and based on a single injection per preparation, differences in peak intensity between the chromatograms should not be interpreted quantitatively.

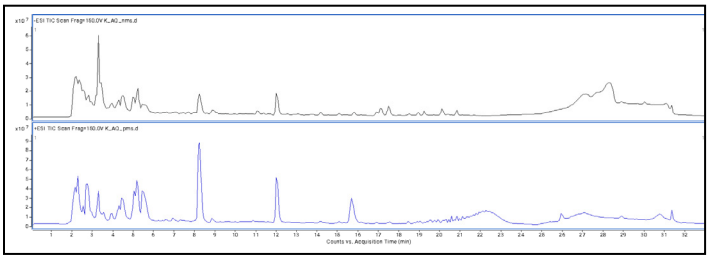


FIGURE 1. Total-ion chromatograms (TIC) of the kefir-derived postbiotic (KP): negative ionization mode (ESI−, upper panel) and positive ionization mode (ESI+, lower panel).

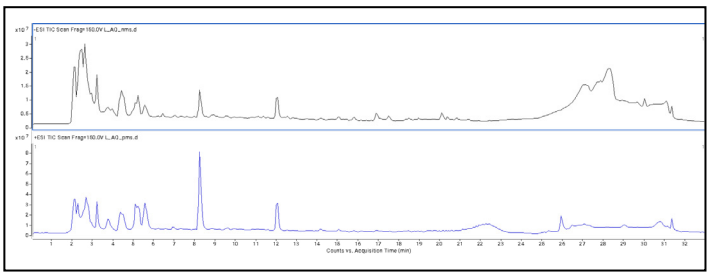


FIGURE 2. Total-ion chromatograms (TIC) of the Lactobacillus-derived postbiotic (LP): negative ionization mode (ESI−, upper panel) and positive ionization mode (ESI+, lower panel).

Antimicrobial activity of kefir-derived and Lactobacillus-derived postbiotics

The antimicrobial activities of kefir-derived postbiotic (KP) and *Lactobacillus*-derived postbiotic (LP) against *Staphylococcus aureus* ATCC43303 and *Candida albicans* ATCC10231 are presented in TABLE II. Both preparations reduced the viable counts of the tested microorganisms after 12 h of exposure.

For *S. aureus*, the initial population of 4.03 ± 0.04 $\log_{10}$ CFU/mL decreased to 1.04 ± 0.03 $\log_{10}$ CFU/mL after treatment with KP, corresponding to a reduction of 2.99 ± 0.04 $\log_{10}$ CFU/mL ($P < 0.001$ versus the initial count). Treatment with LP reduced the viable count below the detection limit of the plating method (<1.00 $\log_{10}$ CFU/mL) in all three experiments, corresponding to a reduction of at least 3.02 ± 0.01 $\log_{10}$ CFU/mL. The reductions achieved by the two preparations against this organism did not differ significantly (P = 0.28). It should be noted, however, that the LP values are minimum estimates: once the counts had fallen below the detection limit the assay could no longer resolve any further difference between the two preparations. The absence of a statistically significant difference against this organism therefore reflects the resolution limit of the enumeration method and should not be interpreted as evidence that the two preparations are equivalent against it.

TABLE II

Antimicrobial effects of kefir-derived (KP) and Lactobacillus-derived (LP) postbiotics on Staphylococcus aureus ATCC43303 and Candida albicans ATCC10231 after 12 h of exposure ($\log_{10}$ CFU/mL)

Microorganism	Postbiotic	Initial count ($\log_{10}$ CFU/mL)	Final count ($\log_{10}$CFU/mL)	Reduction ($\log_{10}$ CFU/mL)
S. aureus ATCC43303	KP	4.03 ± 0.04	1.04 ± 0.03	2.99 ± 0.04[b]
	LP	4.02 ± 0.01	<1.00	>3.02 ± 0.01[b]
C. albicans ATCC10231	KP	5.10 ± 0.03	3.36 ± 0.02	1.74 ± 0.05[c]
	LP	5.11 ± 0.01	<1.00	>4.11 ± 0.01[a]

KP: kefir-derived postbiotic; LP: Lactobacillus-derived postbiotic; ATCC: American Type Culture Collection; CFU: colony-forming units; SD: standard deviation. Postbiotics were mixed 1:1 (v/v) with the pathogen suspension and incubated at 23 °C for 12 h, after which survivors were enumerated by plate counting. Values are means ± SD of three independent experiments (n = 3). Initial count: viable population in the mixture at time zero; final count: viable population after 12 h; reduction: initial minus final count, calculated for each experiment. "<1.00" denotes counts below the detection limit of the plating method; these were assigned the value of the detection limit for the statistical analysis, so the corresponding reductions are conservative minimum values (">"). Reduction values not sharing a superscript letter differ significantly (two-way ANOVA followed by Tukey's test, P < 0.05). The reductions produced by KP and LP did not differ significantly against *S. aureus* (Welch's t-test, P = 0.28), whereas LP produced a significantly greater reduction than KP against *C. albicans* (P < 0.001)

A clearer difference was observed against *C. albicans*. The initial yeast population of 5.10 ± 0.03 $\log_{10}$ CFU/mL decreased to 3.36 ± 0.02 $\log_{10}$ CFU/mL after exposure to KP, corresponding to a reduction of 1.74 ± 0.05 $\log_{10}$ CFU/mL (P < 0.001 versus the initial count). LP, in contrast, reduced the viable count of *C. albicans* below the detection limit (<1.00 $\log_{10}$ CFU/mL) in all three experiments, corresponding to a reduction of at least 4.11 ± 0.01 $\log_{10}$ CFU/mL. Against this organism the difference between the two preparations amounted to approximately 2.4 log10 CFU/mL and was statistically significant (P < 0.001).

The two preparations therefore behaved differently against the two test organisms. Against *S. aureus* both reduced the viable count to, or close to, the detection limit of the method, and the measured reductions could not be distinguished statistically. Against *C. albicans*, by contrast, LP was distinctly the more

effective preparation. Two-way analysis of variance accordingly showed a significant interaction between the test organism and the postbiotic preparation ($P < 0.001$): the relative performance of KP and LP differed between the two organisms. Because the *S. aureus* counts approached the detection limit, however, this interaction may in part reflect the limited measuring range of the assay for that organism rather than a genuine difference in susceptibility.

In the present study both the kefir-derived postbiotic (KP) and the Lactobacillus-derived postbiotic (LP) preparation reduced the viable counts of *Staphylococcus aureus* and *Candida albicans*. However, the extent of inhibition differed between the two preparations, most markedly against *C. albicans*. KP produced a marked antibacterial effect against *S. aureus*, whereas LP reduced both organisms to below the detection limit of the plating method. The difference between the two preparations was statistically significant against *C. albicans* but not against *S. aureus*, where both preparations approached the detection limit and the assay could not resolve a further difference. Under the conditions applied here, an influence of the microbial source on the observed reduction could therefore be demonstrated only against *C. albicans*. Both preparations produced substantial reductions within the 12 h contact time. For KP, the 2.99 $\log_{10}$ drop in *S. aureus* amounts to clearing approximately 99.9% of the population. LP reduced the counts of both organisms to below 1.00 $\log_{10}$ CFU/mL. The difference between the two preparations was greatest against the yeast: KP reduced *C. albicans* by 1.74 $\log_{10}$, while LP achieved at least 4.11 $\log_{10}$, a considerably greater reduction of the yeast than that achieved by the grain-derived preparation.

Metabolite profiles of the two preparations

A broad, late-eluting envelope appearing after ~25 min, during the high-organic wash step of the gradient (90% B), was present in both samples, more prominently in negative mode, and did not correspond to any annotated metabolite. Because this late-eluting region was common to both preparations rather than sample-specific, it most plausibly reflects shared matrix or medium-derived material rather than a discriminating biological metabolite; an uninoculated-medium (MRS) blank was not analysed in the present study, and its analysis would be required to confirm the origin of this region. More broadly, since several annotated species (free amino acids, purine bases and pyroglutamic acid) are also abundant in MRS broth, subtraction of such a blank would be required to distinguish the microbially derived fraction from medium components carried over into the preparations. This is acknowledged as a limitation of the present work.

Shared metabolites and their significance

The eighteen shared metabolites, and in particular the common aromatic and organic acids (3-phenyllactic, hydroxyphenyllactic and succinic acids), may represent a shared chemical signature that could contribute a baseline antimicrobial capacity to both preparations, although, in the absence of a medium blank, part of this shared core may derive from the MRS broth rather than from microbial metabolism. The qualitative differences, acetylated aromatic amino acids and ascorbic acid in KP versus free valine and short peptides in LP, may then modulate this activity.

This shared-core / variable-feature structure suggests that the antimicrobial effect of kefir postbiotics may arise from a metabolite assemblage rather than from any single compound, and that KP and LP may be compositional variants of a common fermentation background rather than chemically distinct products. In applied terms, these shared metabolites may serve as reference markers for the chemical standardization of kefir-based postbiotics intended for use as food-preservative or functional ingredients.

Antimicrobial activity in relation to microbial origin

The antimicrobial activity of postbiotics has been reported to vary depending on the *Lactobacillus* strain, culture conditions (including medium, temperature, and incubation time), as well as the type and concentration of antimicrobial compounds produced [23]. In agreement with this, previous studies have also emphasized that postbiotic efficacy is highly dependent on microbial origin and production conditions [24].

In contrast to the reduction observed here, a previous study reported that postbiotics from the kefir-derived *L. kefiri* Kef-m15 showed no antimicrobial activity against *S. aureus*. The discrepancy probably rests on strain differences and on the production conditions applied there, 72 h at 30 °C [25]. However, other studies have demonstrated that kefir-derived *Lentilactobacillus* kefiri postbiotics possess significant antimicrobial and antibiofilm activity against *S. aureus* and other mastitis-associated pathogens [26].

These effects have been linked to alterations in bacterial hydrophobicity, auto-aggregation, and exopolysaccharide production, leading to reduced biofilm formation and virulence potential [26]. Furthermore, postbiotic applications have been reported to achieve microbial reductions of approximately 1.5–2 $\log_{10}$ CFU/g in different microbial systems [27].

Regarding *Candida* species, postbiotics derived from Lactiplantibacillus plantarum have been shown to reduce the growth of *C. albicans* and *C. kefyr.* Under monoculture conditions, a reduction of approximately 0.11 $\log_{10}$ CFU/mL was observed after 24 hours, while in mixed cultures reductions of 0.62 $\log_{10}$ and 0.64 $\log_{10}$ CFU/mL were reported for *C. albicans* and *C. kefyr,* respectively [28].

In addition, postbiotics derived from *Apilactobacillus kunkeei* have been reported to exhibit inhibitory activity against *C. albicans* [29]. Similarly, postbiotic–chitosan combinations derived from *Pediococcus acidilactici* have been reported to significantly reduce foodborne pathogens, including *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes*, with reductions ranging from 1.58 to 3.21 $\log_{10}$ CFU/mL under cold storage conditions [19]. Likewise, kefir-derived *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei* postbiotics have been shown to inhibit *C. albicans* biofilm formation [30].

These findings indicate that the antimicrobial activity of postbiotics is highly dependent on culture composition and production conditions [24]. Postbiotics are also referred to as cell-free supernatants (CFS), metabolites, or biogenic compounds [5]. Previous studies have demonstrated the antimicrobial potential of probiotic-derived cell-free supernatants.

For example, purified CFS from *Lactobacillus rhamnosus* L60 and Lactobacillus fermentum L23 has been shown to exert inhibitory effects against *Neisseria gonorrhoeae* [31]. Similarly, cell-free fermented supernatants from *Lactobacillus acidophilus*, *Lactobacillus delbrueckii, Lactobacillus johnsonii,* and *Lactiplantibacillus plantarum* have been reported to significantly inhibit the growth of *Pseudomonas aeruginosa strains* [32]. Together, these reports show that probiotic-derived postbiotics act against a broad range of pathogens.

Work on kefir and its products has leaned heavily toward antioxidant and broad antimicrobial effects, while antifungal activity against Candida has drawn far less scrutiny. Even so, *Lactobacillus*-derived postbiotics are known to curb both the planktonic growth and the biofilm formation of *Candida species, C. albicans* among them [10, 30, 33]. In the present study, the significantly higher inhibition of *C. albicans* observed with LP compared to KP is consistent with these findings.

Evaluation of the antimicrobial effect of the kefir postbiotic (KP)

In this study, KP reduced *S. aureus* by 2.99 ± 0.04 $\log_{10}$ CFU/mL within only 12 h of contact, corresponding to the elimination of approximately 99.9% of the bacterial population. Cell-free supernatants of lactic acid bacteria have been shown to inhibit *S. aureus* in a concentration-dependent manner [34]; the present finding suggests that a kefir-derived postbiotic can produce a substantial reduction within a 12 h contact time, although, in the absence of an untreated control and of intermediate time points, neither the kinetics of the effect nor the contribution of the holding period itself can be established.

This reduction is consistent with the organic and aromatic acids annotated in the preparation. Among the shared acids, 3-phenyllactic acid (PLA) acts through a dual mechanism that combines cytoplasmic-membrane permeabilization with direct interaction with genomic DNA [35], and PLA and related aromatic acids display well-documented antifungal activity [36, 37]. Succinic and other weak organic acids contribute pH-dependent inhibition: at low pH the undissociated form crosses the cytoplasmic membrane, dissociates intracellularly and acidifies the cytoplasm, impairing membrane potential, transport and macromolecular synthesis [11].

The smaller reduction of *C. albicans* (1.74 ± 0.05 $\log_{10}$ CFU/mL) is consistent with the layered chitin–β-glucan–mannoprotein wall acting as a diffusion barrier that limits acid penetration [38]; aromatic and phenolic acids may nonetheless increase fungal membrane permeability and promote leakage of cytoplasmic contents, which may contribute to the reduction observed [39]. The KP-specific presence of L-ascorbic acid (an antioxidant) and acetylated aromatic amino acids is consistent with a tentative description of KP as an acid/antioxidant-leaning preparation, although the contribution of these minor components to the antibacterial effect is most likely secondary to that of the organic and aromatic acids.

Evaluation of the antimicrobial activity of the Lactobacillus postbiotic (LP)

LP lowered both *S. aureus* and *C. albicans* to below the detection limit (<1.00 $\log_{10}$ CFU/mL). Against *C. albicans* this represented a significantly greater reduction than that produced by KP, whereas against *S. aureus* the two preparations could not be distinguished statistically, since KP had already reduced the count to close to the detection limit. The metabolite data show that LP shares the principal antimicrobial acids with KP and differs mainly by the presence of free valine and three short peptides (Pro-Leu, Leu-Val and Pro-Val-Leu), indicating a more proteolytically active preparation. It should be emphasised, however, that these particular di-/tripeptides are not established antimicrobial agents: the membrane-permeabilizing peptides characteristic of lactic acid bacteria are bacteriocins, cationic, amphipathic peptides of ~20–60 residues that form pores in the cytoplasmic membrane [40], whereas the small neutral peptides annotated here are better interpreted as markers of proteolysis than as direct effectors of inhibition.

Spermidine, often invoked as an antimicrobial amine, was detected in both KP and LP and therefore cannot account for the greater activity of LP against *C. albicans*. Consequently, the superior performance of LP against *C. albicans* cannot be assigned to any single annotated compound; it is most plausibly explained by a combination of possibly higher concentrations of the shared organic and phenolic acids (which were not quantified in the present study) and proteinaceous or bacteriocin-like components lying outside the targeted small-molecule scope of this analysis.

Consistent with a proteinaceous, non-acid contribution to such activity, an antimicrobial fraction isolated from milk kefir was found to consist principally of amino acids and small peptides (notably alanine, tyrosine and phenylalanine derivatives) rather than bacteriocins or simple organic acids, and its bactericidal activity was strongly pH-dependent [41].

Confirmatory assays, such as testing of pH-neutralized supernatant, proteinase K or heat treatment, and membrane-integrity probes (propidium iodide uptake, leakage of 260-nm-absorbing material), are required to establish the underlying mechanism. These considerations reinforce the need for bioactivity-guided fractionation to attribute postbiotic

antimicrobial activity to specific metabolites, as recently demonstrated for *Lactobacillaceae* postbiotics against antibiotic-resistant Salmonella [42].

Overall, the findings of this study suggest that KP may exert a weaker inhibitory effect, possibly involving acidification, although the pH of the preparations was not measured, whereas LP displays a markedly stronger effect against the yeast. Differences of this kind suggest that postbiotic applications could be tailored to their chemical composition and intended purpose. LP, in particular, may merit further investigation as a candidate for biopreservative applications where yeasts are the organisms of concern, on account of its marked activity against *C. albicans.*

Limitations of the study

Several limitations constrain the interpretation of these results and are stated here explicitly. The LC-MS characterization was qualitative and rested on a single injection of one cell-free preparation per group, so analytical reproducibility was not assessed and the presence or absence of individual features could not be verified across replicates. No uninoculated-medium blank was analysed, which means that medium-derived components cannot be separated from microbially derived metabolites; this applies in particular to the free amino acids, purine bases and pyroglutamic acid of the shared core and to the late-eluting envelope observed after ~25 min.

Because the activated kefir grains were also propagated in MRS broth before the postbiotic was recovered, KP and LP share the same medium background, and a medium-derived contribution to both the chemical profiles and the antimicrobial effects therefore cannot be ruled out. In addition, metabolites were not quantified, so differences in the concentration of the shared acids, which are a plausible explanation for the stronger activity of LP against *C. albicans*, could not be examined.

Accordingly, the compositional comparison presented here is descriptive and provisional, and the mechanistic interpretations offered above are proposed as hypotheses to be tested rather than as established conclusions. A further constraint applies to the antimicrobial data: because LP reduced both test organisms to below the detection limit of the plating method, the reductions attributed to it are minimum values, and the assay was unable to quantify how far beyond that limit its activity extends. A dilution series or a more sensitive enumeration method would be required to establish the true magnitude of the difference between the two preparations, particularly against *S. aureus*, where KP had itself reduced the count to close to the detection limit.

Several further constraints apply to the antimicrobial comparison. First, the design compared the count in the mixture at time zero with the count after 12 h, without a preparation-free control held under the same conditions, a sterile-MRS control or a pH-matched control; the reduction observed can therefore not be attributed in full to the microbial metabolites, since the contribution of the medium components, of the pH of the preparations and of the 12 h holding period itself cannot be separated from it. For this reason the effects reported here should be understood as viable-count reductions under the conditions tested rather than as measures of intrinsic antimicrobial potency. Second, because both preparations were produced in MRS broth, this medium-derived contribution applies to KP and LP alike, and it therefore bears on the antimicrobial comparison as well as on the chemical one. Third, the two preparations were not produced under identical conditions: the activated kefir grains were incubated in MRS broth at 30 °C, whereas the *Lactobacillus* isolates were grown at 37 °C, so that differences between KP and LP may reflect incubation temperature as well as microbial origin. Finally, the two preparations are cell-free filtrates; although such preparations are widely reported as postbiotics and are referred to here by that term, they do not contain the inactivated cells or cell fragments that the ISAPP definition also encompasses, and "cell-free supernatant" describes them more narrowly.

CONCLUSIONS

Both the kefir-derived postbiotic (KP) and the *Lactobacillus*-derived postbiotic (LP) reduced the viable counts of *Staphylococcus aureus* and *Candida albicans* under the conditions tested, and LP reduced both organisms to below the detection limit of the plating method. The difference between the two preparations was statistically significant against the yeast but not against the bacterium, where both preparations approached the detection limit and the assay could not resolve a further difference. The LC-MS profiles of the two preparations overlapped to a large extent: 18 annotated metabolites formed a shared core that included succinic, hydroxyphenyllactic and 3-phenyllactic acids, compounds that may plausibly account for a substantial part of the inhibition observed with either preparation.

The features that distinguished the two preparations were few: ascorbic acid and acetylated aromatic amino acids were detected only in KP, and free valine and short peptides only in LP. None of these compounds, considered individually, accounts for the greater activity of LP against the yeast, and the present data do not allow the antimicrobial effect to be attributed to any single metabolite. It should also be noted that, because the LP counts fell below the detection limit of the plating method, the reductions reported for LP are minimum values and the true difference between the two preparations may be larger than the measured one.

Taken together, these observations suggest that the microbial source of a postbiotic may influence both its chemical composition and its activity against C. albicans, a consideration that may be relevant to the design of postbiotic-based food ingredients. The organic and aromatic acids common to KP and LP could additionally serve as reference markers should kefir-derived postbiotics require chemical standardization.

These conclusions must, however, be read in the light of several methodological limitations that the authors consider substantial. First, the LC-MS characterization was qualitative and was based on a single injection of one cell-free preparation per group; analytical reproducibility was therefore not assessed, and the compositional differences reported here are preliminary rather than established. Second, no uninoculated-medium (MRS) blank was analysed, so the microbially derived fraction of the

metabolite pool could not be distinguished from components carried over from the culture medium; several of the annotated compounds, including free amino acids, purine bases and pyroglutamic acid, are also abundant in MRS broth. Third, and directly related, both preparations were produced in MRS broth, the activated kefir grains having likewise been transferred to MRS, so a common medium-derived contribution to the chemical profiles of KP and LP, and possibly to their antimicrobial effects, cannot be excluded; moreover, the two preparations were incubated at different temperatures (30 °C and 37 °C), so that the differences between them cannot be attributed to microbial origin alone. Fourth, no quantification and no bioactivity-guided fractionation were performed, so the observed activity cannot be assigned to specific metabolites. Finally, the antimicrobial evaluation was restricted to in vitro conditions and to two reference strains.

For these reasons the present findings should be regarded as a preliminary, hypothesis-generating comparison rather than a definitive characterization of kefir-derived postbiotics. Quantitative metabolomics with replicate injections and medium-blank subtraction, bioactivity-guided fractionation, and validation in real food matrices are required before the compositional and functional differences described here can be considered established, and before either preparation can be proposed for practical application.

Conflict of interests

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Supplementary Table S1

Putative LC-MS annotations for the kefir-derived (KP) and Lactobacillus-derived (LP) postbiotics, with molecular formula, ion species, theoretical m/z, retention time (RT) and mass error (Δ, ppm) in each preparation.

Compound	Molecular formula	Ion	Theoretical m/z	RT KP (min)	Δ KP (ppm)	RT LP (min)	Δ LP (ppm)
Shared core (n = 18)							
Spermidine	C7H19N3	[M+H]+	146.1651	1.96	2.06	1.90	3.82
Lysine	C6H14N2O2	[M+H]+	147.1128	2.36	-0.01	2.30	1.35
L-Proline	C5H9NO2	[M+H]+	116.0706	2.83	-0.15	2.83	1.21
Guanine	C5H5N5O	[M+H]+	152.0566	3.57	-1.15	3.77	0.06
L-Methionine	C5H11NO2S	[M+H]+	150.0583	3.90	-3.05	3.90	-2.07
Hypoxanthine	C5H4N4O	[M+H]+	137.0457	4.50	0.41	4.50	1.38
Pyroglutamic acid	C5H7NO3	[M-H]-	128.0353	4.50	1.51	4.51	-0.28
Xanthine	C5H4N4O2	[M-H]-	151.0261	4.98	-0.77	4.98	0.84
L-Isoleucine	C6H13NO2	[M+H]+	132.1019	5.04	0.45	5.11	3.78
L-Tyrosine	C9H11NO3	[M-H]-	180.0666	5.18	0.77	5.24	-0.10
Succinic acid	C4H6O4	[M-H]-	117.0193	5.50	0.11	5.44	-3.16
L-Leucine	C6H13NO2	[M+H]+	132.1019	5.51	4.54	5.58	0.54
Phenylalanine	C9H11NO2	[M-H]-	164.0717	8.26	0.20	8.26	0.32
L-α-Hydroxyisovaleric acid	C5H10O3	[M-H]-	117.0557	11.07	0.59	11.07	-0.90
Tryptophan	C11H12N2O2	[M+H]+	205.0971	11.94	-0.45	12.08	4.87
Hydroxyphenyllactic acid	C9H10O4	[M-H]-	181.0506	12.41	-2.22	12.41	-3.48
N-Acetyl-leucine	C8H15NO3	[M-H]-	172.0979	18.50	-2.24	18.50	0.83
3-Phenyllactic acid	C9H10O3	[M-H]-	165.0557	20.11	-2.82	20.11	-2.64
Detected only in KP (n = 3)							
L-Ascorbic acid	C6H8O6	[M-H]-	175.0248	3.37	-3.17	–	–
Acetyl-L-tyrosine	C11H13NO4	[M-H]-	222.0771	13.08	-2.72	–	–
N-Acetyl-D-phenylalanine	C11H13NO3	[M-H]-	206.0822	20.45	-2.84	–	–
Detected only in LP (n = 4)							
L-Valine	C5H11NO2	[M+H]+	118.0862	–	–	3.23	0.82
L-Prolyl-L-leucine	C11H20N2O3	[M+H]+	229.1547	–	–	9.87	-0.62
L-Leucyl-L-valine	C11H22N2O3	[M+H]+	231.1703	–	–	9.99	1.18
Prolyl-valyl-leucine	C16H29N3O4	[M+H]+	328.2231	–	–	16.36	3.61

Annotations are putative, based on accurate-mass measurement within ±5 ppm (MSI confidence level 3); a single injection of one preparation per group was analysed and the data are qualitative (presence/absence), not quantitative. Accurate mass alone does not resolve structural or stereochemical isomers: L-isoleucine/L-leucine (both C6H13NO2, m/z 132.1019) are distinguished only by retention time, and D/L configurations were not assigned. Ion species: [M+H]+ acquired in positive ESI; [M−H]− in negative ESI. –, not detected.