Metabolomic Approach Assisted High Resolution LC−ESI-MS Based Identification of a Xenobiotic Derivative of Fenhexamid Produced by *Lactobacillus casei*

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Ⓢ *Supporting Information*

ABSTRACT: Fenhexamid is a widely used fungicide with one of the highest maximum tolerance limits approved for fruits and vegetables. The goal of this study was to examine if fenhexamid is metabolized by a nontarget organism, a *Lactobacillus* species (*Lactobacillus casei* Shirota), a probiotic strain of the human gastrointestinal tract. The assignment of bacterial derivatives of the xenobiotic fenhexamid was substantially facilitated by a metabolomic software based approach optimized for the extraction of molecular features of chlorine-containing compounds from liquid chromatography−electrospray ionization−quadrupole time-of-flight mass spectrometry data with an untargeted compound search algorithm. After validating the software with a set of seventeen chlorinated pesticides and manually verifying the result lists, eleven molecular features out of 4363 turned out to be bacterial derivatives of fenhexamid, revealing the *O*-glycosyl derivative as the most abundant one that arose from the fermentation medium of *Lactobacillus casei* Shirota in the presence of 100 μg/mL fenhexamid.

KEYWORDS: xenobiotics, LC−ESI-QTOF-MS, metabolomics, fenhexamid-*O*-glucoside

■ INTRODUCTION

The gastrointestinal tract (GIT) is highly exposed to xenobiotics since compounds from numerous sources can occur within. The intestinal tract is therefore particularly important in toxicology both as a target organ and as a site for absorption of xenobiotics to the organism. The most important xenobiotic source is the oral ingestion of drugs, food components and environmental pollutants.[1] Additionally, reactive metabolites can also be formed through the activity of drug-metabolizing enzymes expressed by intestinal cells, and finally the interaction of xenobiotics with the gut microbiota can also lead to the production of a significant amount of derivatives.[2,3] The microbial activity significantly affects the metabolism and bioavailability of various compounds from external origin, e.g., from food and drug compounds.[4] This microbiome related source should be highlighted since the absorption in the distal gut results in around 10% of the metabolites in the host systemic blood flow from bacterial origin.[5]

Regarding the human GIT, lactic acid bacteria are among the abundant species.[6] The most common genera are *Lactobacillus*, *Streptococcus* and *Enterococcus*. Besides their well established role in the food industry, *Lactobacilli* have gained attention for their probiotic properties[7] and the production of a wide variety of enzymes: intracellular β-galactosidase production of *Lactobacillus acidophilus*,[8] β-glucoronidase-like activity in more *Lactobacillus* species[9−11] and oleuropeinase and β-glucosidase

activities in *Lactobacillus plantarum*[12,13] were already observed in previous studies.

Fruit and vegetable consumption is an important part of a healthy diet, and as a consequence fungicides and their residues on the surface of these products may represent a significant source of xenobiotics and precursors of xenobiotics in the gut. There are huge public concerns about the extensive utilization of pesticides including fungicides since potential harmful effects on human health are attributed to these bioactive chemicals.[14,15] The effects of relevant pesticides on the applied microbiota in dairy and meat production were presented in previous studies, where it was concluded that in general the reproduction of lactic acid bacteria was hindered and their metabolism was also changed (however to different extent).[16,17] It is also noticeable that lactic acid bacteria used in meat production could degrade the pesticides in their environment, which positively influenced the quality of the product.[18]

Concerning the generally applied pesticides, special emphasis should be attributed to fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexane carboxamide; $C_{14}H_{17}Cl_2NO_2$; CAS No. 126833-17-8). Fenhexamid is a low water-soluble hydroxy-anilide fungicide and is applied worldwide in the case of numerous horticultural crops, fruits and

vegetables.[19] According to the actual regulation it is a fungicide with one of the highest maximum tolerance level (MTL)/ maximum residue limit (MRL) values allowed even up to 30 mg/kg (for chervil, chrysanthemum, coriander, corn salad, cress, sorrel, endive, lettuce, orach, parsely, purslane, red chicory, spinach) and 40 mg/kg (for lettuce) in the US and in the EU, respectively. Moreover, there has been a recent opinion published by the European Food Safety Authority (EFSA) allowing the MRL value of fenhexamid to be raised in the case of some fruits.[19]

The information about the effects of fenhexamid on nontarget organisms is extremely scarce even though fenhexamid was introduced in 1998.[20] Facing the high impact of xenobiotics on human microbiota[21] and the occurrence of fenhexamid residues on fruits and vegetables,[22,23] we decided to investigate in vitro the possible detoxification or metabolized products of fenhexamid produced by *L. casei*. However, the assignment of supposedly low abundant arising compounds in complex sample matrices is extremely difficult to carry out without a metabolomic software assisted approach requiring high resolution chromatographic and mass spectrometric techniques (LC−ESI-QTOF-MS or LC−ESI-Orbitrap-MS). This approach has been successfully applied to reveal the metabolomic aspect of the symbiosis of leguminous plants and rhizobial bacteria[24] and to characterize the effect of beta-lactam antibiotics on the microbiome of exposed rat models.[25] Therefore, in order to cover the highest possible number of fenhexamid derived xenobiotics of bacterial origin, our study was based on metabolomic data mining.

■ MATERIALS AND METHODS

Reagents. Fenhexamid was obtained by the chromatographic cleanup of Teldor (Bayern Cropscience, Monheim am Rhein, Germany) commercial pesticide product.[26] Fifteen other, also chlorine containing pesticides (acetamiprid, boscalid, dichlorvos, difenoconazol, epoxiconazole, fenbuconazole, haloxyfop, imazalil, linuron, metconazole, prochloraz, pyraclostrobin, tebuconazole, tetraconazole and thiacloprid) and formic acid were obtained from the Sigma−Aldrich group (St. Louis, MO, USA). Penconazole was ordered from Dr. Ehrenstorfer Gmbh (Augsburg, Germany), and HPLC gradient grade acetonitrile (ACN) and ethanol were ordered from Fisher Scientific (VWR, Radnor, PA, USA). Ultrapure water (>18 MΩ cm⁻¹) was obtained from a Millipore Milli-Q system (Bedford, MA, USA).

Chemicals. Fenhexamid-O-glucoside (purity >98%; CAS No. 1392231-43-4) was synthesized according to the protocol described by Polgár et al.[26] Fenhexamid stock solutions (~4000 mg L⁻¹) were prepared in pure ethanol and were stored at −18 °C until analysis.

Maintenance of the Cell Cultures. *L. casei* Shirota species originated from the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest). The cells were kept on traditional MRS slants and were subcultured before the experiments were conducted to reach the appropriate vitality of the cultures. One hundred microliter volumes of cell cultures were subsequently inoculated into 20 mL MRS broths containing 20.0 g of D-glucose L⁻¹ (pH = 6.5, Himedia M369; Himedialabs, Mumbai, India) at 37 °C. Fenhexamid standard stock solutions sterilized by filtration through 0.22 μm disposable PTFE filters of 4000 μg/mL concentration (25, 200 and 500 μL) were added to the three separate broths (20 mL) to set the 5, 40 and 100 μg/mL final fenhexamid concentrations, respectively. Ethanol concentration was set in all samples to 2.5% v/v. The broths were kept for 72 h at 37 °C. Cell density was determined by OD₆₀₀ measurements using a Thermo Scientific Ecolution 300 EB (Thermo Scientific, Bellefonte, PA, USA) spectrophotometer.

An additional cell-free control sample was also set, containing 100 μg/mL fenhexamid in MRS broth and 2.5% v/v ethanol.

Sample Preparation. Samples were collected in sterile 2.0 mL Eppendorf tubes after 24, 48 and 72 h, and then the cells were separated by centrifugation at 10,000g at 4 °C in a Hettich Mikro 22R centrifuge (Tuttlingen, Germany). Ten fold dilutions were performed on the collected supernatants adjusting a final ACN concentration of 5% v/v. Before injection the samples were filtered through a disposable 0.45 μm PTFE syringe filter.

The remaining ~14 mL samples were stored at −18 °C and used to perform solid phase extraction (SPE) enrichment procedure. The enrichment was performed on Waters Sep-PaK Vac C18 3 cm³ disposable cartridges (Waters, Milford, MA, USA) that were applied on a Thermo Scientific Hypersep 16-port SPE vacuum manifold (Thermo Scientific) equipped with an oil free vacuum pump. For the enrichment 5 mL supernatants were collected by centrifugation from the remaining ~14 mL samples. In order to keep the octadecyl groups of the SPE cartridges unfolded (active), 1 mL of ACN was added to the 5 mL sample leading to 16.7% v/v ACN concentration. Steps of the enrichment process are shown in Table 1 in the Supporting Information.

The concentrated samples were then evaporated under argon flow to total dryness and resuspended in 400 μL of 20% v/v ACN:H₂O. Before injection the samples were filtered through a 0.45 μm PTFE syringe filter.

HPLC−ESI-MS analyses. The HPLC−ESI-MS couplings were carried out with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). 10 μL of the sample was injected on a Zorbax Eclipse XDB-C18 reversed phase column (3.5 μm, 2.1 mm × 50 mm; Agilent). The HPLC flow rate was set to 400 μL/min. For the chromatographic gradient, solution A was water with 0.1% v/v formic acid, and solution B was ACN with 0.1% v/v formic acid. Gradient elution was set as follows: 0−3 min 5% B; 3−7 min up to 9% B; 7−10 min 9% B, 10−20 min up to 20% B; 20−22 min 20% B, 22−40 min up to 100% B; 40−42 min 100% B, 42−43 min down to 5% B; 43−47 min 5% B.

For the full scan screening of the samples, a 6530 Accurate Mass QTOF LC/MS system (Agilent) was applied. The QTOF-MS instrument was operated with an Agilent 6220 instrument derived dual ESI ion source in positive and negative ionization modes. Mass calibration was performed by introducing a mass calibration solution by direct infusion according to the manufacturer's instructions. The instrument performed automatic continuous mass correction by introducing a reference solution. This solution contains internal reference masses purine (C₅H₅N₄/[M + H]⁺/at m/z 121.050873, /[M − H]⁻/at m/z 119.036320) and HP-0921 ([hexakis(1H,1H,3H-tetrafluoropentoxy)phosphazene] (C₁₈H₁₉O₆N₃P₃F₂₄)/[M + H]⁺/at m/z 922.009798, /[M + HCOO]⁻/at m/z 966.000725). Due to the continuous mass correction, mass accuracy was ~3 ppm and mass resolution was above 10,000 (fwhm) at m/z 400. The instrumental parameters and settings are summarized in Table 2 in the Supporting Information.

Data Mining Strategy: Seeking Chlorine Containing Metabolites. The full-scan data recorded was processed with Mass Hunter Qualitative Analysis software (Version B.06.00; Agilent), and untargeted compound search (general unknown screening) was performed by the Molecular Feature Extraction algorithm (MFE). During MFE the software annotates the detected ions to create molecular features. Ions are grouped together to create a given molecular feature whose extracted ion chromatograms (EIC) share similar elution profiles, and additionally their mass differences can be explained via isotope ratios, adduct or dimer formations or neutral losses and in-source fragmentation events. The software takes into consideration user defined parameters regarding mass accuracy, mass resolution, minimal signal and compound intensities, minimum number of ions per compound etc.

According to the known degradation and detoxification pathways of fenhexamid,[27−30] the intermediates and byproducts still possess the chlorine containing residue; therefore searching for chlorinated molecules may give a hint to find fenhexamid related compounds. As chlorine has two abundant isotopes, Cl³⁵ and Cl³⁷, the chlorine containing compounds share a characteristic isotopic pattern with a
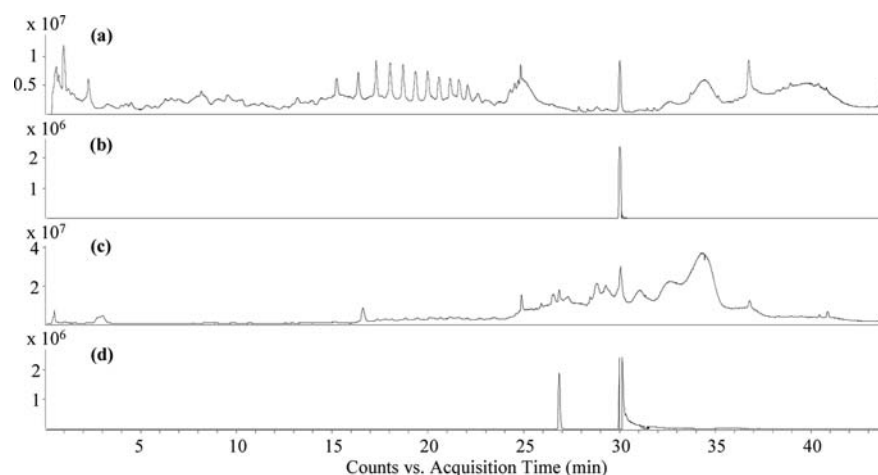
Figure 1. Effect of the cleanup and enrichment performed by SPE. (a) TIC of the supernatant of *L. casei* Shirota collected after 24 h is highly suppressed in positive ion mode, especially when the ACN concentration in the eluent is between 15 and 35% v/v. (b) EIC of fenhexamid eluted at 29.95 min. (c) TIC obtained after SPE clean up and enrichment. (d) The EIC of fenhexamid at 29.95 min indicates saturation due to the enrichment, while an additional peak of fenhexamid also appears at 26.78 min, relating to an in-source fragment of a previously undetected compound.

distinctive, intensive M + 2 isotope. Additionally, there is a mass defect (defined as the difference in exact mass between the masses of the isotopes of an element minus the integer mass difference, where the reference isotope is the monoisotopic mass)[31] caused by Cl^{37} isotope giving an optional remark to the identification of chlorine containing compounds.

By the settings of molecular formula generation at least one chlorine atom was set up as an obligatory element which enabled us to focus only on the chlorine containing compounds and filter out the others. Considering the mentioned marks of chlorine content of the detected compounds, molecular formulas were generated to determine their elemental composition.

In order to find the optimal settings of MFE for assigning chlorine containing metabolites, a 10 ng/mL pesticide standard mix of the 17 different, chlorine containing pesticides was prepared. During optimization, the isotope spacing tolerance parameter was reduced from the default value (5 mDa + 7 ppm) to 2 mDa + 3 ppm. As a result, MFE could assign all of the 17 pesticides correctly by detecting at least four isotopologues per pesticide.

MFE may result in false positive and negative hits even after optimization, consequently each differential chlorine containing compound was verified manually considering the isotopic pattern, mass defect and the shape of correlating EICs.

Differential Expression Analysis. Differential expression analysis of the samples after processing by Mass Hunter Qualitative analysis was performed by Mass Profiler Professional software (Version 2.1.5; Agilent). Among the assigned molecular features the nondifferentiating ones between the experimental and control samples were filtered out by the following statistical methods: filter by frequency, t test ($p <$ 0.01) and fold change analysis (FC ≥ 2).

Identification of Differential Compounds. The compounds of interest were analyzed in a subsequent run on the HPLC−ESI-QTOF-MS instrument in Auto MS/MS mode to perform fragmentation by collision induced dissociation (CID) of the precursor molecules in order to gain structural information. Based on the fragmentation pattern the chemical structure can be deduced or it can be compared with those of authentic standards. The latter is still considered to be the "golden standard" method in compound identification.[32] The user defined parameters of the Auto MS/MS mode are summarized in Table 2 in the Supporting Information.

■ RESULTS

Detection of Chlorine Containing Compounds without Enrichment by MFE. The three different fenhexamid

concentrations in cell cultures were set to determine the relatively highest level of fenhexamid exposure that can be tolerated by *Lactobacilli* and that can also ensure abundant chlorine containing metabolites of bacterial origin. Since bacterial cell density did not show significant difference between the cultures with the three fenhexamid levels applied and between different incubation periods, only the sample with 100 μg/mL fenhexamid addition after 24 h incubation period was analyzed in further experiments. On the other hand, higher fenhexamid concentration could not be justified since 100 μg/mL is already 2.5 times higher than the highest allowed MTL/MRL value for any fruit or vegetable commodity.

The control and experimental samples were analyzed first without enrichment. The MFE process revealed that the most intensive chlorine containing compound in all analyzed samples was fenhexamid: its protonated form, sodium and potassium adducts and the isotopologues of each pseudomolecular ion were also found.

The assignment of chlorine compounds by MFE was followed by differential expression analysis between control and experimental samples. The detected molecular features shared the characteristic chlorine isotope pattern and mass defect. Monoisotopic masses of these molecules other than fenhexamid are as follows: m/z 357.9726, 414.0349 and 470.0975 in positive ionization mode and m/z 601.3972, 714.4806 and 710.3498 in negative ionization mode, respectively. Full scan spectra are presented in Figures 1−6 in the Supporting Information. All the six compounds were detected in the cell-free control sample and in the experimental sample as well, indicating that they derive from the fenhexamid solution itself and not from bacterial origin.

Detection of Chlorine Containing Compounds by MFE after SPE Enrichment. Besides fenhexamid and its byproducts no other metabolites with chlorine content could be detected without enrichment. Consequently, SPE enrichment was applied in order to enhance the concentration of less abundant analytes thus allowing their detection and MS/MS identification. Since the chlorine atom containing ring is not affected by bio- and photodegradation of fenhexamid, these products keep their hydrophobic character to be retained on a reversed phase C18 SPE cartridge, providing the additional

Table 1. Considered and Verified Parameters of the Differential Molecular Features Assigned by the Software in Positive Ion Mode[a]

compd no.	molecular formula score	neutral mass, Da	t_R, min	max height, cps	signal-to-noise ratio	rel isotopic mass defect (M vs M + 2), mDa	ref for identification
1	90.3	822.2097	39.46	6.5×10^3	2.4	0.9	na[b]
2	93.0	878.2724	39.41	3.2×10^4	12.2	0.6	na
3	94.7	766.1465	36.22	2.1×10^4	1.7	2.3	na
4[c]	98.6	380.0355	30.11	6.7×10^3	0.6	3.2	na
5	99.1	463.1176	26.78	1.5×10^6	102.6	2.5	Polgár et al.[26]

[a]Features are sorted by molecular formula score in increasing order. [b]Not applicable. [c]Detected as double charged.
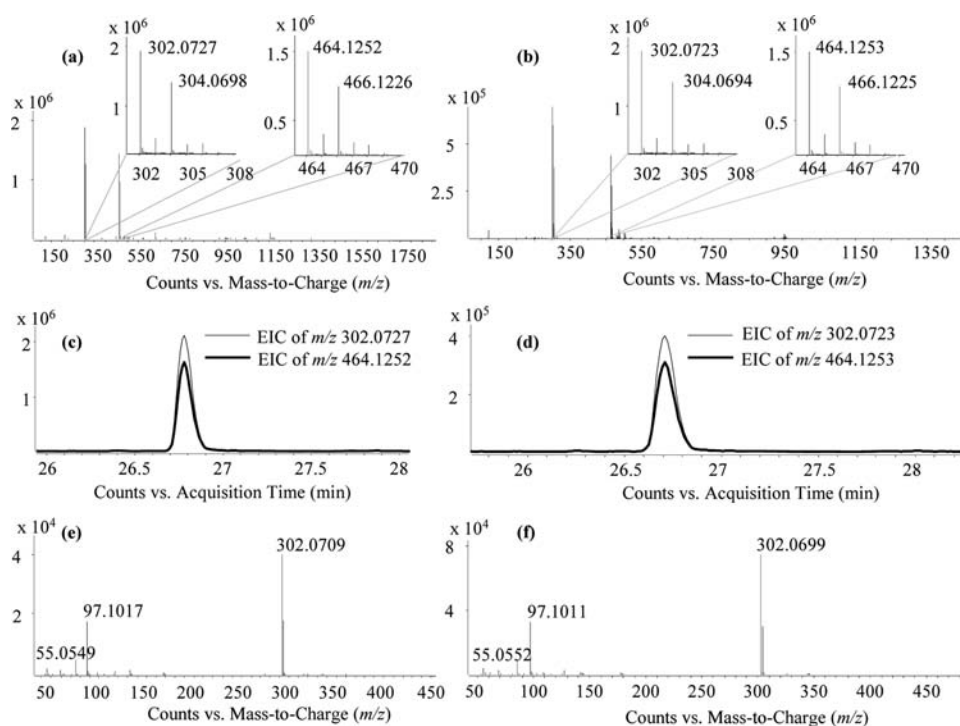

Figure 2. Full scan spectra of *L. casei* sample (a) and 5 μg/mL fenhexamid-*O*-glucoside standard (b) at 26.78 min retention time show great similarity. EIC (positive ion mode) of *m/z* 464.1252 (c) shows that 5 is eluted at 26.78 min, matching the EIC of the same *m/z* value of the standard (d). Product ion spectra (e and f) at the same retention time also show great similarity of the 5 arising from the *L. casei* supernatant and the fenhexamid-*O*-glucoside standard. Based on all of the observations 5 was identified as fenhexamid-*O*-glucoside.

advantage to deplete the sample from the hydrophilic compounds, e.g., salts, and to spare the column and mass spectrometer. As it can be seen on the total ion chromatograms (TIC; Figure 1a,c), the hydrophilic compounds were removed; meanwhile the hydrophobic ones became more intensive than prior to SPE enrichment.

MFE resulted in 2986 and 1377 (altogether 4363) molecular features in positive and negative ion modes, respectively, but the molecular formula generation (MFG) with the preset obligatory chlorine atom content and differential expression reduced the number of assigned compounds to 83 and 47 in positive and negative ion mode, respectively. After the assigned features were sorted by MFG score, the best fits were verified manually.

Among the differential compounds in positive ion mode, five were supposed to contain chlorine, based on the isotopic pattern and molecular formula generation score. Considered parameters of these compounds are summarized in Table 1, and full scan spectra of these compounds are summarized in Figures 7−11 in the Supporting Information.

To decide whether these compounds are truly chlorine containing, the characteristic mass defect of chlorine, signal-to-noise ratio and the raw mass spectra were also checked and evaluated manually in a subsequent step. Among the hits it can be seen that except for 5 the compounds are of low abundance resulting also in low signal-to-noise ratio. In the cases of 1, 3 and 4 the extremely low signal-to-noise ratios make it impossible to prove that the assigned isotope ratio truly originates from chlorine content. Doubts about these features are also enhanced by the fact that the samples are enriched (~12.5× concentration) and the mass accuracy of QTOF instruments are negatively influenced by such low abundance values. Consequently, these molecular features were not considered as chlorine containing metabolites of bacterial origin.

In the case of 2 the signal-to-noise ratio, mass defect and the relatively higher abundance enabled us to decide that it contains chlorine. However, by manual evaluation of the raw mass spectrum at retention time (t_R) 39.4 min the characteristic isotope pattern of the previously detected byproduct having 470.0975 Da monoisotopic mass was recognized. The EICs of

Table 2. Most Probable Chlorine Containing Differential Molecular Features and Their Parameters in Negative Ion Mode[a]

compd no.	molecular formula score	neutral mass, Da	t_R, min	max height, cps	signal-to-noise ratio	rel isotopic mass defect (M vs M + 2), mDa	ref for identification
6	92.7	390.1622	34.23	3.9×10^3	27.5	−12.6	na[b]
7	94.5	229.0502	31.60	2.7×10^3	6.1	1.6	na
8	94.6	417.0818	38.25	2.3×10^3	27	1.1	na
9	97.4	317.0587	29.31	9.2×10^3	72	2.2	na
10	97.8	505.1279	28.35	1.7×10^4	600.7	2.3	na
11	98.2	463.1164	26.78	1.5×10^6	>1000	2.5	Polgár et al.[26]

[a]The molecular features were manually verified in the same manner as in positive ion mode. [b]Not applicable.

their m/z values showed strong correlation suggesting that **2** is an additional form of the mentioned fenhexamid byproduct and its presence is not the result of any bacterial activity. The EICs of their monoisotopic masses can be seen in Figure 12 in the Supporting Information.

5 (m/z 464.1252) was the most abundant compound showing high signal-to-noise ratio, and its mass defect was also appropriate (Figure 2a). By examining the mass spectrum at t_R = 26.78 min fenhexamid was also recognized at m/z 302.0727, however t_R of fenhexamid is 29.95 min (Figure 1b,d). EICs of the peaks showed strong correlation so based on the observation it was proved that fenhexamid is present as the in-source fragment of **5** (Figure 2b). The difference between the mass-to-charge ratios of **5** and fenhexamid, 162.053, is characteristic of a neutral loss of a glucose moiety. These observations served as important factors in structure elucidation since a characteristic substructure could be identified.

Possible elemental compositions were generated considering the isotopic pattern, the in-source fragment (fenhexamid; $C_{14}H_{18}Cl_2NO_2^+$; $[M^+H]^+$) derived information and the accurate mass data being $C_{20}H_{28}Cl_2NO_7^+$ ($[M^+H]^+$; theoretical m/z 464.1238, Δppm = 3.32) as the most probable record differing in $C_6H_{10}O_5$, a glycoside moiety, from fenhexamid. Indeed, the glucoside conjugate, fenhexamid-O-glucoside, is one of the detoxification derivatives of fenhexamid, detected in various plants.[15,19,33,34] On the other hand, this molecule has never been identified from any direct bacterial metabolism, although glycosylation of chemical compounds by bacterial activity is an already known process that is carried out mainly for stabilization or solubilization purposes.[35,36]

In order to verify if **5** is identical to fenhexamid-O-glucoside, its standard solution was analyzed in a concentration of 5 μg/mL. The retention time, the in-source fragmentation and the product ion data matched those of **5** (Figure 2c,d). Additionally, MS/MS data of the **5** (m/z 302.0695 (100); 97.1011 (41); 55.0549 (6)) and the fenhexamid-O-glucoside standard (m/z 302.0699 (100); 97.1011 (43); 55.0552 (5)) also showed high matching (Figure 2e,f).

The spiking experiment was evaluated also for the estimation of the amount of the O-glycosylated derivative. Without taking into account either the possible analyte losses during the cleanup procedure or intracellular residues, approximately 0.1% of fenhexamid was converted into fenhexamid-O-glucoside. This conversion rate is significantly less than in the case of plant detoxification where more than 24% was observed.[26] This observation indicates that the actual bacterial glycosylation event does not belong to typical bacterial detoxification pathways, where specific and nonspecific enzymatic activities of hydrolase and mono- and dioxygenases are usually involved.[37,38]

In negative ion mode, the process of seeking chlorine containing compounds was carried out in the same manner as in positive mode. After differential expression, six molecular features (**6−11**) detailed in Table 2 were verified manually. Their full scan spectra are summarized in Figures 13−18 in the Supporting Information.

As can be seen in Table 2, **6−9** compared to **11** were of very low intensity, consequently their identification was also hindered. Considering the fact that negative ionization mode is more sensitive than positive, especially after SPE enrichment, the low abundant compounds were not subjected to identification. The only abundant differentiating compound was **11** that was the same as **5** in positive ion mode, as can be seen in Figure 19 in the Supporting Information, referring to fenhexamid-O-glucoside.

The signal-to-noise ratio of **10** (m/z 504.1211) was high enough to carry out MS/MS fragmentation. As presented in Figure 20 in the Supporting Information, the fragment of fenhexamid could be assigned in the MS/MS spectrum and could also be detected in the EIC of the MS/MS spectra of fenhexamid and fenhexamid-O-glucoside, indicating this compound is also a derivative of fenhexamid. As the intensity and mass accuracy of the MS/MS fragments were not adequate for unambiguous identification and no previously reported metabolites of fenhexamid could be matched,[39] this bacterial derivative could not be identified.

Although the bacterial formation of fenhexamid-O-glucoside has been unambiguously proven in the applied MRS medium by *L. casei*, it requires further investigation if this process does occur in the GIT in order to address bioavailability and toxicity issues. On the other hand, this study calls attention to the observation that actual metabolomic data treatment processes may still require additional validation procedures and manual verification to considerably decrease false positive and negative hits.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Positive and negative full scan spectra of molecular features of various monoisotopic masses, positive full scan spectra of **1−5**, negative full scan spectra of **6−11**, EICs, negative TIC, table showing steps of SPE enrichment of *Lactobacillus* supernatants, and table showing instrumental parameters of the HPLC−ESI-MS setups addressed in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ACN, acetonitrile; CID, collision induced dissociation; EFSA, European Food Safety Authority; EIC, extracted ion chromatogram; ESI, electrospray ionization; GIT, gastrointestinal tract; LC, liquid chromatography; MFE, molecular feature extraction algorithm; MFG, molecular formula generation; MRL, maximum residue limit; MRS, de Man, Rogosa, Sharpe; MTL, maximum tolerance limit; QTOF, quadrupole time-of-flight; t_R, retention time; SPE, solid phase extraction; TIC, total ion chromatogram

■ REFERENCES

(1) Sambruy, Y.; Ferruzza, S.; Ranaldi, G.; De Angelis, I. Intestinal cell culture models: Applications in toxicology and pharmacology. *Cell Biol. Toxicol.* **2001**, *17*, 301−317.

(2) Kent, T. H.; Cardelli, R. M.; Stamler, F. W. Small intestinal ulcers and intestinal flora in rats given indomethacin. *Am. J. Pathol.* **1969**, *54*, 237−249.

(3) Niu, X.; De Graaf, I. A. M.; Groothuis, G. M. M. Evaluation of the intestinal toxicity and transport of xenobiotics utilizing precision-cut slices. *Xenobiotica* **2013**, *43*, 73−83.

(4) Possemiers, S.; Grootaert, C.; Vermeiren, J.; Gross, G.; Marzorati, M.; Verstraete, W.; Van de Wiele, T. The intestinal environment in health and disease - Recent insights on the potential of intestinal bacteria to influence human health. *Curr. Pharm. Des.* **2009**, *15*, 2051−2065.

(5) Wikoff, W. R.; Anfora, A. T.; Liu, J.; Schultz, P. G.; Lesley, S. A.; Peters, E. C.; Siuzdak, G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3698−3703.

(6) Whitman, W. B.; Coleman, D. C.; Wiebe, W. J. Prokaryotes: The unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6578−6583.

(7) Chaikham, P.; Apichartsrangkoon, A.; Jirarattanarangsri, W.; Van de Wiele, T. Influence of encapsulated probiotics combined with pressurized longan juice on colon microflora and their metabolic activities on the exposure to simulated dynamic gastrointestinal tract. *Food Res. Int.* **2012**, *49*, 133−142.

(8) Choonia, H. S.; Lele, S. S. Beta-Galactosidase release kinetics during ultrasonic disruption of Lactobacillus acidophilus isolated from fermented Eleusine coracana. *Food Bioprod. Process.* **2011**, *89*, 288−293.

(9) McConnell, M. A.; Tannock, G. W. A note on lactobacilli and beta-glucuronidase activity in the intestinal contents of mice. *J. Appl. Bacteriol.* **1993**, *74*, 649−651.

(10) Pham, P. L.; Dupont, I.; Roy, D.; Lapointe, G.; Cerning, J. Production of exopolysaccharide by Lactobacillus rhamnosus R and analysis of its enzymatic degradation during prolonged fermentation. *Appl. Environ. Microbiol.* **2000**, *66*, 2302−2310.

(11) Russell, W. M.; Klaenhammer, T. R. Identification and cloning of gusA, encoding a new beta-glucuronidase from Lactobacillus gasseri ADH. *Appl. Environ. Microbiol.* **2001**, *67*, 1253−1261.

(12) Marsilio, V.; Lanza, B.; Pozzi, N. Progress in table olive debittering: Degradation in vitro of oleuropein and its derivatives by Lactobacillus plantarum. *J. Am. Oil Chem. Soc.* **1996**, *73*, 593−597.

(13) Ciafardini, G.; Marsilio, V.; Lanza, B.; Pozzi, N. Hydrolysis of oleuropein by Lactobacillus plantarum strains associated with olive fermentation. *Appl. Environ. Microbiol.* **1994**, *60*, 4142−4147.

(14) Golden, R.; Kimbrough, R. Weight of evidence evaluation of potential human cancer risks from exposure to polychlorinated biphenyls: an update based on studies published since 2003. *Crit. Rev. Toxicol.* **2009**, *39*, 299−331.

(15) European Parliament. Regulation (EC) No. 396/2005 of the European Parliament and of the Council on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Off. J. Eur. Union* **2005**, *16*, 1−16.

(16) Ayana, I. A. A. A.; El Deen, A. A. G.; El-Metwally, M. A. Behavior of certain lactic acid bacteria in the presence of pesticides residues. *Int. J. Dairy Sci.* **2011**, *6*, 44−57.

(17) Clair, E.; Linn, L.; Travert, C.; Amiel, C.; Séralini, G. E.; Panoff, J. M. Effects of roundup (R) and glyphosate on three food microorganisms: Geotrichum candidum, Lactococcus lactis subsp. cremoris and Lactobacillus delbrueckii subsp. bulgaricus. *Curr. Microbiol.* **2012**, *64*, 486−491.

(18) Abou-Arab, A. A. K. Degradation of organochlorine pesticides by meat starter in liquid media and fermented sausage. *Food Chem. Toxicol.* **2002**, *40*, 33−41.

(19) EFSA. Reasoned opinion on the modification of the existing MRLs for fenhexamid in currants and beans with pods. *EFSA J.* **2013**, *11*, 3110.

(20) Russell, P. E. A century of fungicide evolution. *J. Agric. Sci.* **2005**, *143*, 11−25.

(21) Johnson, C. H.; Patterson, A. D.; Idle, J. R.; Gonzalez, F. J. Xenobiotic metabolomics: Major impact on the metabolome. *Annu. Rev. Pharmacol. Toxicol.* **2012**, *52*, 37−56.

(22) Kmellár, B.; Abrankó, L.; Fodor, P.; Lehotay, S. J. Routine approach to qualitatively screening 300 pesticides and quantification of those frequently detected in fruit and vegetables using liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Addit. Contam., Part A* **2010**, *27*, 1415−1430.

(23) Angioni, A.; Schirra, M.; Garau, V. L.; Melis, M.; Tuberoso, C. I. G.; Cabras, P. Residues of azoxystrobin, fenhexamid and pyrimethanil in strawberry following field treatments and the effect of domestic washing. *Food Addit. Contam.* **2004**, *21*, 1065−1070.

(24) Zhang, N.; Venkateshwaran, M.; Boersma, M.; Harms, A.; Howes-Podoll, M.; Den Os, D.; Ané, J. M.; Sussman, M. R. Metabolomic profiling reveals suppression of oxylipin biosynthesis during the early stages of legume-rhizobia symbiosis. *FEBS Lett.* **2012**, *586*, 3150−3158.

(25) Zheng, X.; Xie, G.; Zhao, A.; Zhao, L.; Yao, C.; Chiu, N. H. L.; Zhou, Z.; Bao, Y.; Jia, W.; Nicholson, J. K.; Jia, W. The footprints of gut microbial-mammalian co-metabolism. *J. Proteome Res.* **2011**, *10*, 5512−5522.

(26) Polgár, L.; García-Reyes, J. F.; Fodor, P.; Gyepes, A.; Dernovics, M.; Abrankó, L.; Gilbert-López, B.; Molina-Díaz, A. Retrospective screening of relevant pesticide metabolites in food using liquid chromatography high resolution mass spectrometry and accurate-mass databases of parent molecules and diagnostic fragment ions. *J. Chromatogr., A* **2012**, *1249*, 83−91.

(27) Cabras, P.; Farris, G. A.; Pinna, M. V.; Pusino, A. Behavior of a fenhexamid photoproduct during the alcoholic fermentation of Saccharomyces cerevisiae. *J. Agric. Food Chem.* **2004**, *52*, 8053−8056.

(28) Maheswari, M. A.; Lamshöft, M.; Sukul, P.; Spiteller, P.; Zühlke, S.; Spiteller, M. Photochemical analysis of 14C-fenhexamid in aqueous solution and structural elucidation of a new metabolite. *Chemosphere* **2010**, *81*, 844−852.

(29) Abbate, C.; Borzí, D.; Caboni, P.; Baglieri, A.; Gennari, M. Behavior of fenhexamid in soil and water. *J. Environ. Sci. Health, Part B* **2007**, *42*, 843−849.

(30) FAO. *Pesticide residues in food − 2005*; Food and Agriculture Organization of the United Nations: Rome, 2005.

(31) Thurman, E. M.; Ferrer, I. The isotopic mass defect: A tool for limiting molecular formulas by accurate mass. *Anal. Bioanal. Chem.* **2010**, *397*, 2807−2816.

(32) Xiao, J. F.; Zhou, B.; Ressom, H. W. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *TrAC, Trends Anal. Chem.* **2012**, *32*, 1−14.

(33) EFSA. Modification of the existing MRL for fenhexamid in onions. *EFSA J.* **2010**, *8*, 1696−1718.

(34) EFSA. Modification of the existing MRLs for fenhexamid in various leafy vegetables. *EFSA J.* **2010**, *8*, 1455−1475.

(35) Hyung Ko, J.; Gyu Kim, B.; Joong-Hoon, A. Glycosylation of flavonoids with a glycosyltransferase from Bacillus cereus. *FEMS Microbiol. Lett.* **2006**, *258*, 263−268.

(36) Kim, G. E.; Kang, H. K.; Seo, E. S.; Jung, S. H.; Park, J. S.; Kim, D. H.; Kim, D. W.; Ahn, S. A.; Sunwoo, C.; Kim, D. Glucosylation of the flavonoid, astragalin by Leuconostoc mesenteroides B-512FMCM dextransucrase acceptor reactions and characterization of the products. *Enzyme Microb. Technol.* **2012**, *50*, 50−56.

(37) Zhang, H.; Yang, C.; Li, C.; Li, L.; Zhao, Q.; Qiao, C. Functional assembly of a microbial consortium with autofluorescent and mineralizing activity for the biodegradation of organophosphates. *J. Agric. Food Chem.* **2008**, *56*, 7897−7902.

(38) Karpouzas, D. G.; Singh, B. K., Microbial Degradation of Organophosphorus Xenobiotics: Metabolic Pathways and Molecular Basis. In *Adv. Microb. Physiol.*, Robert, K. P., Ed.; Academic Press: 2006; Volume 51, pp 119−225.

(39) Anderson, C.; Brumhard, B.; Ditgens, K.; Reiner, H. Metabolism of fenhexamid (KBR 2738) in plants, animals, and the environment. *Pflanzenschutz-Nachr. Bayer (Engl. Ed.)* **1999**, *52*, 227−251.