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Metabolomic and Lipidomic Characterization of *Oxalobacter formigenes* strains HC1 and OxWR by UHPLC-HRMS

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Abstract

Diseases of oxalate, such as nephrolithiasis and primary hyperoxaluria, affect a significant portion of the United States population and have limited treatment options. *Oxalobacter formigenes*, an obligate oxalotrophic bacterium in the mammalian intestine, has generated great interest as a potential probiotic or therapeutic treatment for oxalate-related conditions due to its ability to degrade both exogenous (dietary) and endogenous (metabolic) oxalate, lowering the risk of hyperoxaluria/hyperoxalemia. Although all oxalotrophs degrade dietary oxalate, *Oxalobacter* is the only species shown to initiate intestinal oxalate secretion to draw upon endogenous, circulating oxalate for consumption. Evidence suggests that *Oxalobacter* regulates oxalate transport proteins in the intestinal epithelium using an unidentified secreted bioactive compound, but the mechanism of this function remains elusive. It is essential to gain an understanding of the biochemical relationship between *Oxalobacter* and the host intestinal epithelium for this microbe to progress as a potential remedy for oxalate diseases. This investigation includes the first profiling of the metabolome and lipidome of *Oxalobacter formigenes*, specifically the human strain HC1 and rat strain OxWR, the only two strains shown thus far to initiate net intestinal oxalate secretion across native gut epithelia. This study was performed using untargeted and targeted metabolomics and lipidomics methodologies utilizing ultra-high performance liquid chromatography-mass spectrometry. We report our findings that the metabolic profiles of these strains, although largely conserved, show significant differences in their expression of many compounds. Several strain-specific features were also detected. Discussed are trends in the whole metabolic profile as well as in individual features, both identified and unidentified.

Graphical Abstract

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Keywords

Metabolomics; Lipidomics; *Oxalobacter formigenes*; Mass Spectrometry; LC-MS; Oxalate; Nephrolithiasis; Hyperoxaluria

Introduction

Nephrolithiasis afflicts nearly 1 in 11 people in the United States [1]. Approximately 80% of kidney stones are made of calcium oxalate (CaOx) and form when oxalate (Ox^{2-}) from diet or glyoxylate metabolism in the liver precipitates in the kidneys with calcium to form CaOx stones [2-4]. Recurrence occurs in up to 50% of patients within 5 years of initial onset, and the annual cost of treatment exceeds \$10 billion [5]. In addition to kidney stone disease, there are other oxalate-derived conditions such as Primary Hyperoxaluria (PH), the most common and deadly variant being Primary Hyperoxaluria Type 1 (PH1) [6]. PH1 arises from a mutation in the *AGXT* gene coding for Alanine-Glyoxylate Aminotransferase (AGT), which functions to convert glyoxylate to glycine in the liver [7]. Mutation of *AGXT* results in the retargeting of AGT from its native location, the peroxisome, to the mitochondria, leaving the detoxifying glyoxylate-to-glycine pathway inhibited and forcing excessive glyoxylate-to- Ox^{2-} bioconversion [8]. Chronic hyperoxaluria/hyperoxalemia leads to recurrent stone formation, systemic oxalosis, and, eventually, end-stage renal disease and premature death unless a liver-kidney transplant is performed [6, 7]. Hence, there is much interest in improving current treatment methods for PH and other Ox^{2-} -related ailments.

Research in the field has recently turned to the intestinal microbiome in the search for novel treatments for Ox^{2-} diseases [7]. A class of intestinal bacteria known as oxalotrophs degrade Ox^{2-} in the intestine, reducing dietary Ox^{2-} absorption and, consequently, circulating and urinary oxalate levels, both which directly influence CaOx stone formation [9]. One unique bacterium in this class is the commensal, non-pathogenic *Oxalobacter formigenes* (*Oxf*), a Gram-negative, obligate anaerobic, specialist oxalotroph that exclusively utilizes Ox^{2-} as its sole energy source [10]. While all oxalotrophs degrade dietary Ox^{2-} , *Oxf* has the unique ability to initiate intestinal Ox^{2-} secretion, drawing upon circulating Ox^{2-} in the bloodstream and transporting it into the intestine for consumption [11, 12]. This allows *Oxf* to reduce the Ox^{2-} load in the body to a greater capacity than other oxalotrophs since it is able to access and degrade both endogenous (metabolic) and exogenous (dietary) Ox^{2-} . Compelling evidence suggests *Oxf* initiates intestinal Ox^{2-} secretion through the use of an unknown secreted bioactive compound, or “secretagogue,” hypothesized to regulate Ox^{2-} transport proteins in the intestinal epithelium [11]. To date, the mechanism behind this

function has yet to be described. To elucidate the biochemical nature of the *Oxf*-host relationship, it is essential to first characterize the complete metabolic profile of *Oxf*. We addressed this need by performing metabolomic and lipidomic profiling of two strains of *Oxf*: the human strain HC1, and the rat strain OxWR, using ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS). Currently, HC1 and OxWR are the only two strains of *Oxf* shown to initiate net intestinal Ox^{2-} secretion across native gut epithelia [11, 12]. Gaining an understanding of the biochemical profile of this bacterium and its interaction with the intestinal epithelium, as well as the unique qualities of each strain, is critical if *Oxf* or its metabolic products will ever be used as a potential probiotic or therapeutic treatment for Ox^{2-} diseases. *This study represents the first investigation of the metabolome and lipidome of Oxf and provides the foundation for future work focused on identifying its secreted oxalate-regulating compound.*

Methods

Cell Culture, Harvest, and Lysis

Pure, anaerobic cultures of HC1 and OxWR were grown from frozen glycerol stocks at 37°C in 75mL of previously published *Oxf*-specific media supplemented with 100mM oxalate [10]. To generate sufficient cell mass, 6 bottles of culture were grown to form each replicate (n=4 per strain). During all steps of cell harvest and lysis, samples were kept on ice or under refrigeration, wherever possible. Once sufficient growth was achieved, cultures were combined into replicates and cells were pelleted by centrifugation at 15,180×g, 4°C for 5 min. Supernatants were discarded and pellets were washed by resuspension in 6mL lysis buffer containing 100mM KH_2PO_4 and 1mM MgCl_2 . Cells were again pelleted by centrifugation and supernatants were discarded. Excess lysis buffer was removed and pellets were weighed for downstream normalization. Pellets were resuspended in 3mL lysis buffer and transferred to 15mL polypropylene (PP) vials in a salted ice slurry water bath for sonication. Sonication was performed using a Sonic Dismembrator Model 500 with a Branson Sonicator Probe (Thermo Fisher Scientific, Waltham, MA, USA) by the following method: 30% amplitude for 30 sec, 1 min cool-down, 60% amplitude for 30 sec, 2 min cool-down, 60% amplitude for 15 sec. After sonication, lysates were vortexed, transferred to 1.6mL PP vials, and immediately frozen at -80°C. Lysates were later portioned into 100μL aliquots for individual experimental use to prevent sample alteration from repeated freeze-thaw.

Metabolite Extraction

Metabolites were extracted using 8:1:1 acetonitrile:methanol:acetone by a modified version of our previously reported protein precipitation method [13]. Samples were kept on ice and away from light at all steps, wherever possible. All solvents used were LC-MS grade and obtained from Thermo Fisher Scientific (Waltham, MA). Lysates were normalized pre-extraction to 53mg/mL cell mass per sample, adjusting volume with lysis buffer kept from the cell harvest. Extraction blanks were included to remove exogenous features resulting from the extraction process, replacing sample volume with lysis buffer. All samples from HC1 and OxWR, along with extraction blanks, were simultaneously extracted. To 100μL normalized cell lysate, 20μL of internal standard mix - BOC-L-Tyrosine, BOC-D-

Phenylalanine, N- α -BOC-L-Tryptophan, 2 μ g/ μ L each in 0.1% formic acid - was added followed by brief vortexing. For protein precipitation, 800 μ L 8:1:1 acetonitrile:methanol:acetone was added, samples were vortexed for 15 sec and incubated on ice for 30 min. Protein was pelleted by centrifugation at 20,000 $\times$ g (4°C) for 10 min. Supernatants (750 μ L) containing metabolite content were transferred to new PP tubes and dried under nitrogen at 30°C. Lyophilized metabolite extracts were reconstituted in 100 μ L of 0.1% formic acid in water. Samples were vortexed for 15 sec, incubated on ice for 20 min, and again centrifuged at 20,000 $\times$ g (4°C) for 10 min to remove any remaining particulate. Supernatants (50 μ L) were transferred to glass LC vials and moved to a refrigerated (4°C) Dionex autosampler for analysis.

Lipid Extraction

Lipids were extracted using 4:2:1 chloroform:methanol:water by a modified version of the Folch method [14]. Lysates were again normalized pre-extraction to 53 mg/mL cell mass per sample, adjusting volume with lysis buffer kept from the cell harvest. Extraction blanks were included. All samples from HC1 and OxWR, along with extraction blanks, were simultaneously extracted. To 100 μ L normalized cell lysate, 20 μ L of internal standard mix - LPC(17:0), PC(17:0/17:0), PG(14:0/14:0), PE(15:0/15:0), PS(14:0/14:0), TG(15:0/15:0/15:0) PI(8:0), SM(d18:1/17:0), CER(d18:1/17:0), DG(14:0/14:0), CL(15:0(3)-16:1) SO(d17:1), PAzePC, CER(Glycosyl(β) C12), 14:0 BMP (S,R), LSM(d17:1), 1000ppm each (except PI (8:0) at 250ppm and CL(15:0(3)-16:1) at 100ppm) in 2:1 chloroform:methanol - was added followed by brief vortexing. Next, 400 μ L methanol, then 800 μ L chloroform were added to each sample with brief vortexing after adding each reagent. Samples were incubated on ice for 20 min with vortexing every 10 min. After incubation, 200 μ L water was added, and samples were incubated on ice for 10 min with vortexing every 5 min. Samples were centrifuged at 3,260 $\times$ g, 4°C for 10 min to separate the organic and aqueous layers. From the organic (bottom) layer containing lipid content, 800 μ L was removed and transferred to a new 12mL glass vial on ice. The aqueous layer was re-extracted by adding 400 μ L 2:1 chloroform:methanol, incubating on ice for 10 min, and centrifugation at 3,260 $\times$ g, 4°C for 10 min. From the new organic layer, 400 μ L was removed and combined with the original organic layer. Lipid extracts were dried under nitrogen at 30°C, reconstituted in 300 μ L isopropanol containing injection standards - LPC(10:0), PC(19:0/19:0), PG(17:0/17:0), PE(17:0/17:0), PS(17:0/17:0), TG(17:0/17:0/17:0), 1000ppm each - and centrifuged at 3,260 $\times$ g, 4°C for 10 min for residual protein removal. From each sample, 250 μ L was transferred to glass LC vials for analysis: 200 μ L for each sample + 50 μ L per sample combined into a pooled sample for each strain (pooled samples necessary for lipid identification). LC vials were placed in a refrigerated Dionex autosampler for analysis.

Analytical Instrumentation and Methodology

Analysis by UHPLC-HRMS was performed on a Thermo Q Exactive Orbitrap Mass Spectrometer with heated electrospray ionization paired with a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, MA). Reverse phase chromatography with gradient elution was employed for both metabolite and lipid analyses. For metabolites, the column used was an ACE Excel 2 C18-PFP column (100mm $\times$ 2.1mm, 2.0 μ m) (Advanced Chromatography Technologies, Ltd, Scotland). Solvent A was 0.1% formic acid in water,

solvent B was acetonitrile. The flow rate was 0.35mL/min. Gradient elution was performed as such: 0 - 3 min: 100% A, 3 - 13 min: 100% → 20% A, 13 - 16.5 min: 20% A, 16.5 - 20 min: 100% A at 0.6mL/min (column flush & equilibration). Full scan analysis was performed at 35,000 resolution with scan range 70-1000 m/z in both positive and negative ion mode. Injection volume was 10 μ L for both polarities. For lipids, the column used was an AQUITY UPLC BEH C18 column (50mm $\times$ 2.1mm, 1.7 μ m) (Waters Corporation, Milford, MA, USA) preceded by a corresponding VanGuard pre-column (Waters Corporation, Milford, MA, USA). Solvent A was 60:40 acetonitrile:water with 0.1% formic acid and 10mM ammonium formate, solvent B was 90:8:2 isopropanol:acetonitrile:water with 0.1% formic acid and 10mM ammonium formate. The flow rate was 0.5 mL/min. Gradient elution was performed as such: 0 - 1 min: 80% A, 1 - 3 min: 80% → 70% A, 3 - 4 min: 70% → 55% A, 4 - 6 min: 55% → 40% A, 6 - 8 min: 40% → 35% A, 8 - 10 min: 35% A, 10 - 15 min: 35% → 10% A, 15 - 17 min: 10% → 2% A, 17 - 18 min: 2% A, 18 - 19 min: 2% → 80% A, 19 - 23 min: 80% A (column flush & equilibration). Full scan analysis was performed at 35,000 resolution with scan range 200-2200 m/z in both positive and negative ion mode. Injection volume was 10 μ L for both polarities. We employed iterative exclusion analysis using pooled samples from HC1 and OxWR replicates to increase detection of lower abundance lipid species [15]. For this purpose, a total of 7 rounds of data-dependent MS/MS analysis with successive exclusion of detected features were performed in both positive and negative ion mode at 70,000 resolution, scan range 200-2200 m/z , and 10 μ L injection volume.

Data Processing and Quality Control

Data was quality-checked by examining the performance of spiked internal standards in all samples throughout the sequence. Standards confirmed reproducibility in the analysis showing a relative standard deviation <10%. Data file format conversion was performed using RawConverter[16] (metabolite data) or MSConvert [17, 18] (lipid data). MZmine 2 was utilized for peak picking, chromatographic alignment, and feature identification (metabolites) using our custom internal library by m/z -retention time matching [19]. Non-detected species (intensity = 0) were replaced with half the minimum value in the dataset for statistical purposes [20]. A feature-by-feature signal intensity filtering algorithm was conducted to remove features with significant signal contribution from the background [21]. LipidMatch workflow software, which compares fragmentation m/z values with *in-silico* fragmentation libraries of over 500,000 lipid species, was used for lipid data processing from file conversion to feature identification [21].

Statistical Analysis

MetaboAnalyst 4.0 was used for statistical analysis and figure generation [22]. For statistical analysis, data were normalized to total ion current to correct for instrumental and technical variation and autoscaled to allow a more direct comparison between features of greatly varying intensities [23, 22]. All p-values were determined using the two-tailed, unpaired Student's t-test assuming equal variance on the normalized, scaled dataset. The Bonferroni-Holm false discovery rate (FDR) correction was applied for all determinations of statistical significance [24], which we define with p-value threshold of 0.001.

Results and Discussion

Metabolomics Analysis

A total of 2122 features were detected between positive and negative ion analysis for the HC1 and OxWR metabolomes. As expected, their metabolomes were found to be generally conserved, but many significant differences were identified. Figure 1 shows that distinct separation of the metabolomes of these strains was observed using Principal Component Analysis (PCA) with 69.5% of the variance explained in 2 PCs. 548 features (25.8%) showed a statistically-significant difference in their intensity between strains. The distribution of these significant features between strains was fairly even with 297 features (54.2%) higher expressed in HC1 and 251 features higher expressed in OxWR (Fig. 2). A total of 89 metabolites were identified by *m/z*-retention time matching with our internal library created from pure analytical standards (see Electronic Supplementary Material (ESM) Table S1), 26 of which were higher expressed in HC1 and 63 in OxWR. Table 1 displays information for the top-25 statistically significant identified metabolites, the most significant being deoxycytidine ($p=3.50E-7$) with an 18.68-fold higher intensity in OxWR. Being that deoxycytidine is a nucleoside present in DNA consisting of cytosine and deoxyribose, we initially thought that this finding could be originating from differential deoxycytidine/cytosine content of the genomes of HC1 and OxWR, potentially influencing the relative amounts of specific free nucleosides. However, after examining the genome of HC1 compared to another *Oxf* strain that has been sequenced, the overall size and content of the genomes of *Oxf* strains appears to be too conserved to be responsible for a difference of this magnitude [25, 26]. The metabolite with the largest difference between strains by magnitude was guanosine-3',5'-cyclic monophosphate (cGMP) ($p=4.56E-06$, 1.30E+03-fold higher intensity in OxWR). cGMP is a nucleoside derived from guanosine triphosphate and functions as a secondary messenger [27]. Its function in signal transduction in eukaryotes has been well-studied for some time, but only recently has its importance in bacteria generated interest [28]. Currently, the exact role of cGMP in bacterial signaling is poorly understood [29]. Nevertheless, studies have shown that, for some microbes, cGMP is essential to form cysts [30], and for others, its close-relative cyclic diguanosine monophosphate is involved in the formation and dissolution of biofilms [31]. A fold-difference of this magnitude between two strains of the same species of bacteria is quite compelling. The biological nature of this difference deserves further investigation. Given that deoxycytidine and cGMP showed such varying expression between strains, they were further characterized by MS/MS using CFM-ID 2.0. Interestingly, many of the other significant identified metabolites were also found to be nucleosides, nucleotides, or their derivatives, examples being isocytosine ($p=1.80E-06$, 2.44-fold higher intensity in OxWR), cytidine ($p=7.11E-06$, 2.58-fold higher intensity in OxWR), guanosine ($p=8.26E-06$, 1.30-fold higher intensity in OxWR), and 2'-deoxyadenosine ($p=1.24E-04$, 2.89-fold higher intensity in OxWR). A variety of N-acetylated species also had a significant difference between strains, including N-acetylglutamic acid ($p=6.70E-07$, 6.42-fold higher intensity in OxWR), N-acetylputrescine ($p=2.37E-06$, 2.24-fold higher intensity in OxWR), N-acetylarginine ($p=3.54E-05$, 0.38-fold higher intensity in HC1), and N-acetylaspartic acid ($p=7.80E-05$, 0.55-fold higher intensity in HC1). Table 2 shows the top-25 significant features overall, identified or unidentified. Predictive IDs were assigned to 2 significant

unknowns by exact mass using metabolomics databases and formula prediction tools such as METLIN[32]. The Human Metabolome Database [33], and CEU Mass Mediator [34], although further confirmation of these potential IDs by analysis of analytical standards is necessary. We isolated 17 features deemed to be strain-specific as they were exclusively detected in either HC1 or OxWR (Table 3). Predictive IDs were assigned by exact mass using metabolomics databases for 5 of these 17 features, but further confirmation is required. The presence of strain-specific features, as well as the significant difference in the expression of many shared features, suggests the possibility of evolutionary differences in the metabolic profile of these microbes for survival in the unique environments of the human versus rat intestine. Additional work is needed to clarify the biology behind these observed differences.

Lipidomics Analysis

A total of 2110 features were detected between positive and negative ion analysis for the HC1 and OxWR lipidomes. As with their metabolomes, the lipidomes of HC1 and OxWR were found to be generally conserved, but many significant differences were identified. Figure 3 shows that distinct separation of the lipidomes of these strains was observed by PCA with 63.7% of the variance explained in 2 PCs. 365 features (17.3%) showed a statistically-significant difference in their intensity between strains. Regarding the distribution of these significant lipid features between strains, 250 (68.5%) were higher expressed in OxWR and 115 were higher expressed in HC1 (Fig. 4), a more uneven distribution than was seen with the metabolomics data. A total of 97 unique lipid species across 11 classes were identified by MS/MS fragment m/z matching using LipidMatch (ESM Table S2). Due to the high degree of overlap in lipid detection by MS/MS, possible, albeit less likely, alternative identifications for some species are provided in Table S3 (see ESM). Over one-third of the identified lipids were odd-chain species, which are well-known to be produced by bacteria in the intestinal microbiome [35]. The balance of a bacterium's production of even versus odd-chain fatty acids (as well as straight versus branched-chain species) is due to the substrate specificity of the FabH enzyme [36], which catalyzes the initial condensation reaction that starts the fatty acid elongation cycle [37]. FabH in some bacteria, such as *Escherichia coli* (*E. coli*) and *Streptococcus pneumoniae*, favors acetyl-CoA from intermediary metabolism, resulting in primarily even-chain fatty acid production [38, 39]. Contrarily, FabH in bacteria such as *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus aureus* favors branched-chain acyl-CoA primers from amino acids, resulting in predominantly odd-chain fatty acids [40, 41, 36]. Based on this analysis showing a greater prevalence of even-chain lipids, it would be logical to hypothesize that if *Oxf* possessed FabH for lipid synthesis, it would favor the former-mentioned pathway. It is worth mentioning that, to our knowledge, the role of FabH in *Chef* lipid synthesis has not been characterized. Figure 5 shows a comparison of the lipid profiles for HC1 vs OxWR, taking the sum of the intensities of all identified lipids in each class and displaying as a measure of relative total expression. The profiles for HC1 and OxWR were found to be generally conserved with OxWR containing a slightly higher proportion of PG and BMP species. It is likely that this profile represents the outer and inner (cytoplasmic) membranes of *Oxf* as most of these species fall into classes typically seen in membranes of gram-negative bacteria, such as PE's, PG's, CL's, PS's, and others [42, 43]. Lipid classes with the highest

abundance were PE's, CL's, and DMPE's, together accounting for 90% of the lipid profile in both strains. It is expected to see PE's and CL's in high abundance as these are among the predominant lipid classes in gram-negative cytoplasmic and outer membranes [44, 45]. The relatively high expression of DMPE's is interesting as only a subset of bacteria possess this lipid class within their membranes [46]. It was originally thought that DMPE's were rarely present in bacteria since they were not found in lipid characterizations of *E. coli*, the Gram-negative bacterium model, or *B. subtilis*, the Gram-positive model [47]. However, more recent studies have shown that they exist in a variety of types of eubacteria [48, 49]. Examining specific differences between HC1 and OxWR, Table 4 lists the top-25 most significant identified lipids. PE(37:1) and BMP(17:1/19:1) were found to be the two most significant identified lipids. The majority of the top-significant lipids were PE species, suggesting that these strains have a high degree of heterogeneity even within the most common lipid classes. We did not identify any strain-specific lipids, although this does not rule out their existence. Further studies are needed to determine the biology behind the differences observed between the HC1 and OxWR lipidomes.

Conclusions

We conclude that the metabolomes and lipidomes of *Oxf* strains HC1 and OxWR, although largely conserved, exhibit notable differentiation in terms of relative expression of individual metabolic products with a significant difference in 25.8% of metabolites and 17.3% of lipids detected. Additional work is needed to determine the biological basis of the differential expression of these compounds as well as any unique strain-specific function gained as a result. Although we successfully characterized 89 metabolites and 97 lipids between HC1 and OxWR, we acknowledge that there are many compounds yet to be studied in the *Oxf* metabolic profile. Future investigation focused on identifying unknown features is needed to expand the defined metabolome and lipidome of *Oxf*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMP	Bis(Monoacylglycero)Phosphate
CER	Ceramide
CL	Cardiolipin
CoQ10	Coenzyme Q10

DG	Diacylglycerol
DMPE	Dimethylphosphatidylethanolamine
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LSM	Lysosphingomyelin
OxPE	Oxidized Phosphatidylethanolamine
PAzePC	1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SM	Sphingomyelin
SO	Sphingosine

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Figure 1:
PCA depicts clear separation between the metabolomes of *Ox/HC1* and *OxWR* with 69.5% of the variance explained in 2 PCs and 91.2% explained in 5 PCs.

Figure 2:

Volcano plot shows distribution of significant metabolite features by strain separated by significance ($p = 1E-03$) and magnitude of intensity difference (fold-difference ≥ 1). All 548 significant features met these criteria with 297 (54.2%) and 251 features showing higher expression in HC1 and OxWR, respectively. Top-5 significant identified metabolites: ^aDeoxycytidine, ^bN-Acetylglutamic Acid, ^cIsocytosine, ^dN-Acetylputrescine, ^eGuanosine-3', 5'-Cyclic Monophosphate

Figure 3:
PCA depicts clear separation between the lipidomes of *Oxf*HC1 and OxWR with 63.7% of the variance explained in 2 PCs and 92.1% explained in 5 PCs.

Figure 4:

Volcano plot shows distribution of significant lipid features by strain separated by significance ($p = 1E-03$) and magnitude of intensity difference (fold-change ≥ 1). All 365 significant features met these criteria with 115 and 250 features showing higher expression in HC1 and OxWR, respectively. Top-5 significant identified lipids: ^aPE(31:1), ^bBMP(17:1/19:1), ^cPE(39:4), ^dPE(29:2), ^ePE(37:2)

Figure 5: Distribution of identified lipid species by class intensity sum for HC1 vs OxWR. Largely similar profiles were seen between these strains with a slight increase in the presence of PG and BMP species in OxWR.

Top-25 significant identified metabolites. Detection: Polarity and adduct. ID methods: 1 = m/z match, 2 = retention time match to internal library, 3 = MS/MS fragmentation spectrum match to in silico database. Intensity: Peak area avg ± std dev. Fold-Difference: Magnitude of Intensity Fold-Difference Significance: p 1E-03.

Table 1:

Metabolite Species	m/z	Detection	ID Method	HC1 Intensity	OxWR Intensity	Fold-Difference	Higher Expression	p-value
Deoxycytidine	228.09 77	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2,3	5.08E+ 04 ± 8.50E+ 03	9.99E+ 05 ± 5.19E+ 04	18.68	OxWR	3.50 E-07
N-Acetylglutamic Acid	188.05 64	ESI(−), [M-H]ESI(−), [M-H] [−]	1,2	1.71E+ 06 ± 1.40E+ 05	1.27E+ 07 ± 9.50E+ 05	6.42	OxWR	6.70 E-07
Isocytosine	112.05 08	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	1.16E+ 07 ± 4.65E+ 05	4.00E+ 07 ± 1.97E+ 06	2.44	OxWR	1.80 E-06
N-Acetylputrescine	131.11 79	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	3.09E+ 06 ± 1.76E+ 05	1.00E+ 07 ± 5.74E+ 05	2.24	OxWR	2.37 E-06
Guanosine-3',5'-Cyclic Monophosphate	346.05 45	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2,3	1.09E+ 04 ± 1.88E+ 03	1.41E+ 07 ± 9.00E+ 05	1.30E+03	OxWR	4.56 E-06
Cytidine	244.09 26	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	8.64E+ 06 ± 4.80E+ 05	3.10E+ 07 ± 1.90E+ 06	2.58	OxWR	7.11 E-06
Dihydroxyacetone Phosphate	168.99 01	ESI(−), [M-H]ESI(−), [M-H] [−]	1,2	2.31E+ 06 ± 1.12E+ 05	8.83E+ 05 ± 1.01E+ 05	1.61	HC1	7.65 E-06
Guanosine	282.08 42	ESI(−), [M-H]ESI(−), [M-H] [−]	1,2	2.62E+ 06 ± 1.14E+ 05	6.01E+ 06 ± 4.36E+ 05	1.30	OxWR	8.26 E-06
Ribose	173.04 26	ESI(+), [M+Na] ⁺ ESI(−), [M+Na] ⁺	1,2	1.36E+ 07 ± 5.00E+ 05	2.42E+ 07 ± 1.03E+ 06	0.79	OxWR	8.61 E-06
4-Oxoproline	128.03 53	ESI(−), [M-H]ESI(−), [M-H] [−]	1,2	2.77E+ 07 ± 1.58E+ 06	6.33E+ 07 ± 4.51E+ 06	1.29	OxWR	9.07 E-06
Thiamine Monophosphate	345.07 79	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	2.74E+ 06 ± 1.30E+ 05	5.81E+ 06 ± 3.41E+ 05	1.12	OxWR	1.03 E-05
Phosphoenolpyruvic Acid	166.97 50	ESI(−), [M-H]ESI(−), [M-H] [−]	1,2	2.02E+ 06 ± 9.43E+ 04	4.22E+ 06 ± 3.29E+ 05	1.09	OxWR	1.50 E-05
N-Acetylarginine	217.12 93	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	3.10E+ 07 ± 8.39E+ 05	2.25E+ 07 ± 1.55E+ 06	0.38	HC1	3.54 E-05
Aspartic Acid	134.04 48	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	9.07E+ 06 ± 8.34E+ 04	1.31E+ 07 ± 7.80E+ 05	0.44	OxWR	4.35 E-05
Cysteine	122.02 71	ESI(+), [M+H] ⁺ ESI(−), [M+H] ⁺	1,2	4.03E+ 07 ± 1.02E+ 06	2.89E+ 07 ± 2.92E+ 06	0.39	HC1	5.36 E-05
S-5'-Adenosymethionine	399.14 42	ESI(+), [M+H] ⁺	1,2	3.07E+ 06 ± 1.08E+ 05	6.13E+ 06 ± 4.59E+ 05	1.00	OxWR	5.66 E-05
Glutamic Acid	146.04 58	ESI(−), [M-H] [−]	1,2	7.00E+ 06 ± 4.75E+ 05	1.82E+ 07 ± 1.65E+ 06	1.60	OxWR	5.82 E-05
N-Acetylaspartic Acid	174.04 07	ESI(−), [M-H] [−]	1,2	6.41E+ 07 ± 2.73E+ 06	4.14E+ 07 ± 3.31E+ 06	0.55	HC1	7.80 E-05
5-Oxoproline	130.04 99	ESI(+), [M+H] ⁺	1,2	4.15E+ 07 ± 2.58E+ 06	9.04E+ 07 ± 7.22E+ 06	1.18	OxWR	7.87 E-05
Phosphocholine	218.03 35	ESI(−), [M+Cl] [−]	1,2	1.09E+ 06 ± 3.95E+ 04	5.14E+ 05 ± 9.41E+ 04	1.11	HC1	8.76 E-05
2,4-Dihydroxypyridine	165.04 08	ESI(+), [M+H] ⁺	1,2	2.64E+ 06 ± 1.37E+ 05	1.52E+ 06 ± 2.30E+ 05	0.74	HC1	8.96 E-05
Phosphoserine	186.01 61	ESI(+), [M+H] ⁺	1,2	4.10E+ 05 ± 1.72E+ 05	2.00E+ 06 ± 1.76E+ 05	3.88	OxWR	1.24 E-04
2'-Deoxyadenosine	252.10 89	ESI(+), [M+H] ⁺	1,2	4.28E+ 05 ± 7.72E+ 04	1.66E+ 06 ± 2.35E+ 05	2.89	OxWR	1.24 E-04
3-Dehydroshikimic Acid	171.02 98	ESI(−), [M-H] [−]	1,2	4.05E+ 05 ± 4.82E+ 04	5.88E+ 04 ± 2.88E+ 04	5.89	HC1	1.25 E-04
2-Phosphoglyceric Acid	184.98 53	ESI(−), [M-H] [−]	1,2	1.09E+ 06 ± 1.18E+ 05	2.33E+ 06 ± 1.75E+ 05	1.14	OxWR	1.43 E-04

Table 2:

Top-25 significant metabolite features, including unidentified species. Detection: Polarity and/or adduct. ID methods: 1 = m/z match to metabolomics database or internal library, 2 = retention time to internal library, 3 = MS/MS fragmentation spectrum match to in silico database. Predicted formulas given with ppm error between measured and theoretical mass. Intensity: Peak area avg ± std dev. Fold-Difference: Magnitude of Intensity Fold-Difference. Significance: p 1E-03.

m/z	Detection	ID Method	Predicted Formula	Predicted ID	HC1 Intensity	OxWR Intensity	Fold-Difference	Higher Expression	P-value
566.079&566.079&d	ESI(-), [M+FA-H]-ESI(-), [M+FA-H]-	1	C14H25N3O14P2 (1ppm)C14H25 N3O14P2 (1ppm)	4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythriol	3.03E +06 ± 8.85E +04	2.85E +02 ± 0.00E +00	1.06E+ 04	HC1	3.4 5E-08
267.1317	ESI(+)	N/A	N/A	N/A	1.33E +04 ± 9.16E +03	1.14E +06 ± 4.57E +04	84.51	OxWR	5.3 4E-08
420.1474&420.1474d	ESI(+)	N/A	N/A	N/A	5.42E +02 ± 0.00E +00	3.08E +06 ± 9.83E +04	5.68E +03	OxWR	5.3 4E-08
217.1182&217.1182d	ESI(+), [M+H]+E S(+), [M+H]+	1	C9H16N2O4 (0ppm)C9H16N 2O4 (0ppm)	ψ-Glutamyl-ψ-Aminobutyraldehyde cThr→ProcT hr←Pro	1.61E +07 ± 5.91E +05	5.42E +02 ± 0.00E +00	2.97E+ 04	HC1	5.3 4E-08
617.126&a,d61 7.126&a,d	ESI(-)	N/A	N/A	N/A	1.39E +07 ± 2.38E +05	2.85E +02 ± 0.00E +00	4.86E +04	HC1	1.3 0E-07
404.0509	ESI(-)	N/A	N/A	N/A	5.97E +05 ± 1.87E +04	4.17E +06 ± 2.04E +05	5.98	OxWR	1.3 0E-07
332.0519	ESI(-)	N/A	N/A	N/A	6.34E +04 ± 1.13E +04	7.83E +05 ± 2.00E +04	11.36	OxWR	2.0 5E-07
187.0495	ESI(+)	N/A	N/A	N/A	1.07E +07 ± 2.71E +05	4.79E +06 ± 3.14E +05	1.23	HC1	2.0 5E-07
134.0448	ESI(+)	N/A	N/A	N/A	1.08E +07 ± 4.03E +05	4.02E +05 ± 6.27E +04	25.80	HC1	2.0 5E-07
442.1566	ESI(-)	N/A	N/A	N/A	4.33E +04 ± 1.32E +04	1.27E +06 ± 8.34E +04	28.37	OxWR	2.6 1E-07
595.6633	ESI(-)	N/A	N/A	N/A	8.53E +03 ± 3.47E +03	1.20E +06 ± 7.32E +04	139.35	OxWR	2.7 5E-07
180.0406	ESI(+)	N/A	N/A	N/A	7.09E +03 ± 2.43E +03	4.84E +05 ± 2.26E +04	67.25	OxWR	2.7 5E-07
382.0261	ESI(-)	N/A	N/A	N/A	2.91E +04 ± 1.58E +04	6.86E +05 ± 3.91E +04	22.60	OxWR	2.7 5E-07
238.1525	ESI(+)	N/A	N/A	N/A	2.91E +06 ± 8.83E +04	1.02E +05 ± 5.24E +04	27.48	HC1	2.7 5E-07
619.1412&a,d	ESI(+)	N/A	N/A	N/A	4.32E +06 ± 2.26E +05	5.42E +02 ± 0.00E +00	7.97E +03	HC1	2.7 5E-07
300.0841	ESI(+)	N/A	N/A	N/A	8.92E +05 ± 2.12E +04	3.02E +04 ± 1.99E +04	28.57	HC1	2.7 5E-07
438.5837	ESI(-)	N/A	N/A	N/A	1.04E +06 ± 1.04E +05	2.18E +07 ± 1.04E +06	1993	OxWR	3.0 2E-07
278.1127	ESI(+)	N/A	N/A	N/A	1.05E +05 ± 5.35E +04	3.04E +06 ± 1.55E +05	28.03	OxWR	3.5 0E-07
228.0977	ESI(+), [M+H]+	1,2,3	Deoxyxytidine		5.08E +04 ± 8.50E +03	9.99E +05 ± 5.19E +04	18.68	OxWR	3.5 0E-07
274.0931	ESI(-)	N/A	N/A	N/A	1.98E +07 ± 7.80E +05	1.64E +08 ± 1.12E +07	7.27	OxWR	3.5 4E-07
572.1496	ESI(-)	N/A	N/A	N/A	2.85E +05 ± 3.32E +04	1.81E +06 ± 5.74E +04	5.36	OxWR	4.5 1E-07
179.0339	ESI(+)	N/A	N/A	N/A	1.25E +06 ± 6.80E +04	5.98E +04 ± 1.38E +04	19.96	HC1	4.7 9E-07
310.0696	ESI(-)	N/A	N/A	N/A	3.22E +04 ± 6.41E +03	8.02E +05 ± 5.45E +04	23.87	OxWR	4.7 9E-07
221.1130	ESI(+)	N/A	N/A	N/A	1.44E +05 ± 6.83E +03	7.64E +05 ± 4.43E +04	4.30	OxWR	4.7 9E-07
180.1237	ESI(+)	N/A	N/A	N/A	9.08E +05 ± 2.07E +04	6.76E +04 ± 3.87E +04	12.44	HC1	5.2 4E-07

a,bRegarded to be same feature detected in both pos and neg ion mode. cAmino acid sequence interchangeable. dStrain-specific feature; raw intensity = 0 for 1 strain and was replaced with half minimum value in dataset (pos=5.42E+02, neg=2.85E+02), leading to high fold-difference.

Table 3:

Strain-specific features, all currently unidentified. Detection: Polarity and/or adduct. ID methods; 1 = m/z match to metabolomics database. Predicted formulas given with ppm error between measured and theoretical mass.

<i>Oxf StrainO xf Strain</i>	<i>m/z</i>	Detection	ID Method	Predicted Formula	Predicted ID
HC1	177.0605	ESI(+), [M+2H] ²⁺ -ESI(+), [M+2H] ²⁺	1	C11H20N4O7S (3ppm)C11H20N4O7 S (3ppm)	cSer←Ser←Cys←GlycSer←Ser←Cys←Gly
HC1	217.1182	ESI(+), [M+H]-ESI(+), [M+H] ⁺	1	C9H16N2O4 (0ppm)C9H16N2O4 (0ppm)	ψ-Glutanyl-ψ-Aminobutyraldehyde cThr←ProcThr←Pro
HC1	224.0329	ESI(-), [M+Cl]-ESI(-), [M+Cl] ⁻	1	C7H11NO5 (1ppm)C7H11NO5 (1ppm)	Glutaryl-glycine 2-Amino-6-oxoheptanedioate
HC1	269.4839	ESI(+)	N/A	N/A	N/A
HC1	303.5239	ESI(+)	N/A	N/A	N/A
HC1	357.0260	ESI(+)	N/A	N/A	N/A
HC1	566.0795, 566.0795a 568.0937, 568.0937a	ESI(-), [M4-FA-H]-ESI(-), [M+FA-H] ⁻	1	C14H25N3O14P2 (1ppm)C14H25N3O14P2 (1ppm)	4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol
HC1	617.1265, 617.1265b 619.1412, 619.1412b	ESI(-)	N/A	N/A	N/A
HC1	657.0882	ESI(+)	N/A	N/A	N/A
OxWR	218.5599	ESI(+)	N/A	N/A	N/A
OxWR	227.5652	ESI(+)	N/A	N/A	N/A
OxWR	374.0333	ESI(+)	N/A	N/A	N/A
OxWR	380.1545	ESI(+)	N/A	N/A	N/A
OxWR	415.1688	ESI(+)	N/A	N/A	N/A
OxWR	420.1474	ESI(+)	N/A	N/A	N/A
OxWR	436.1216	ESI(+)	N/A	N/A	N/A
OxWR	510.1436	ESI(-), [M+Cl] ⁻	1	C18H29N5O8S (1ppm)	cCys←Glu←Pro←Gln cAsp←Met←Asn←Pro

a,bRegarded to be same feature detected in both pos and neg ion mode. cAmino acid sequence interchangeable.

Table 4:

Top-25 significant identified lipids. Detection: Polarity and adduct. ID methods: 1 = Data-Dependent (top 5) MS/MS fragment m/z match, 2 = All-Ion-Fragmentation MS/MS fragment m/z match, 3 = Headgroup m/z match (class-ID). Intensity: Peak area avg $\pm$ std dev. Fold-Difference: Magnitude of Intensity Fold-Difference. Expression: Strain with greater avg intensity. Significance: $p \leq 1E-03$.

Lipid Species	m/z	Detection	ID Method	HC1 Intensity	OxWR Intensity	Fold-Difference	Higher Expression	p-value
PE(37:1)	760.5858	ESI(+), [M+H] ⁺	2,3	2.31E+0 7 $\pm$ 3.09E+0 5	9.72E+0 + $\pm$ 8.85E+0 5	1.37	HC1	1.43E-06
BMP(17:1/19:1) ^a	792.5746	ESI(+), [M+NH4] ⁺	1	1.24E+0 + $\pm$ 7.54E+0 5	2.30E+0 8 $\pm$ 1.46E+0 7	0.86	OxWR	1.43E-06
PE(39:4)	782.5679	ESI(+), [M+H] ⁺	2,3	1.39E+0 6 $\pm$ 4.31E+0 4	5.78E+0 5 $\pm$ 7.15E+0 4	1.41	HC1	9.08E-06
PE(29:2)	646.4449	ESI(+), [M+H] ⁺	3	8.20E+0 5 $\pm$ 3.01E+0 4	2.31E+0 6 $\pm$ 1.81E+0 5	1.82	OxWR	1.64E-05
PE(37:2) ^a	758.5695	ESI(+), [M+H] ⁺	2,3	2.61E+0 8 $\pm$ 4.33E+0 6	1.28E+0 8 $\pm$ 1.23E+0 7	1.04	HC1	1.82E-05
BMP(16:0/19:1) ^a	780.5744	ESI(+), [M+NH4] ⁺	1	1.44E+0 8 $\pm$ 2.01E+0 6	1.95E+0 8 $\pm$ 1.45E+0 7	0.35	OxWR	2.03E-05
PE(35:1) ^a	732.5543	ESI(+), [M+H] ⁺	2,3	5.12E+0 9 $\pm$ 7.04E+0 7	3.61E+0 9 $\pm$ 2.71E+0 8	0.42	HC1	2.40E-05
PE(38:2) ^a	772.5856	ESI(+), [M+H] ⁺	2,3	2.83E+0 9 $\pm$ 3.62E+0 7	1.67E+0 9 $\pm$ 1.77E+0 8	0.69	HC1	2.53E-05
PE(14:0/16:0) ^a	664.4913	ESI(+), [M+H] ⁺	1,2	1.42E+0 8 $\pm$ 2.87E+0 6	1.89E+0 8 $\pm$ 1.12E+0 7	0.33	OxWR	3.18E-05
PE(14:0/14:0) ^a	636.4603	ESI(+), [M+H] ⁺	1	5.80E+0 6 $\pm$ 1.42E+0 5	8.87E+0 6 $\pm$ 6.48E+0 5	0.53	OxWR	3.44E-05
BMP(19:1/19:1) ^a	820.6070	ESI(+), [M+NH4] ⁺	1	4.50E+0 7 $\pm$ 4.75E+0 5	6.58E+0 7 $\pm$ 5.54E+0 6	0.46	OxWR	3.51E-05
PE(37:3)	756.5544	ESI(+), [M+H] ⁺	2,3	2.51E+0 6 $\pm$ 4.25E+0 4	1.48E+0 6 $\pm$ 1.68E+0 5	0.70	HC1	6.13E-05
PE(39:2)	786.6014	ESI(+), [M+H] ⁺	2,3	4.22E+0 + $\pm$ 4.12E+0 4	3.16E+0 + $\pm$ 2.81E+0 5	0.34	HC1	1.29E-04
PE(35:2) ^a	730.5379	ESI(+), [M+H] ⁺	2,3	2.81E+0 8 $\pm$ 9.95E+0 6	1.84E+0 8 $\pm$ 1.06E+0 7	0.53	HC1	1.95E-04
PS(14:0/18:1) ^a	734.4975	ESI(+), [M+H] ⁺	1	4.52E+0 6 $\pm$ 2.52E+0 5	8.03E+0 6 $\pm$ 9.60E+0 5	0.78	OxWR	2.29E-04
Co(Q10)	880.7182	ESI(+), [M+NH4] ⁺	1,2	1.48E+0 6 $\pm$ 2.23E+0 5	2.96E+0 6 $\pm$ 3.04E+0 5	1.00	OxWR	2.58E-04
PE(26:0)	608.4300	ESI(+), [M+H] ⁺	3	7.45E+0 5 $\pm$ 2.02E+0 4	9.28E+0 5 $\pm$ 8.08E+0 4	0.25	OxWR	2.66E-04
PE(36:3)	742.5384	ESI(+), [M+H] ⁺	2,3	5.65E+0 6 $\pm$ 9.82E+0 4	8.15E+0 + $\pm$ 8.67E+0 5	0.44	OxWR	2.73E-04
CL(32:0)(38:2) ^a	1451.068 7	ESI(+), [M+NH4] ⁺	1	4.91E+0 7 $\pm$ 5.55E+0 6	1.94E+0 7 $\pm$ 2.77E+0 6	1.53	HC1	2.75E-04
CL(38:2)(38:2)	1531.130 0	ESI(+), [M+NH4] ⁺	1	3.20E+0 + $\pm$ 4.76E+0 5	6.89E+0 5 $\pm$ 1.91E+0 5	3.65	HC1	2.92E-04
PS(17:1/18:1) ^a	774.5287	ESI(+), [M+H] ⁺	1	3.47E+0 7 $\pm$ 1.04E+0 6	6.81E+0 7 $\pm$ 9.78E+0 6	0.96	OxWR	3.72E-04
CL(32:1)(36:2) ^a	1421.022 2	ESI(+), [M+NH4] ⁺	1	9.06E+0 7 $\pm$ 4.82E+0 6	6.15E+0 7 $\pm$ 7.03E+0 6	0.47	HC1	4.13E-04

Lipid Species	<i>m/z</i>	Detection	ID Method	HC1 Intensity	OxWR Intensity	Fold-Difference	Higher Expression	p-value
DG(16:0/17:1)	598.5417	ESI(+), [M+NH ₄] ⁺	1,2	4.75E+0 6 ± 1.15E+0 5	3.68E+0 + ± 2.92E+0 5	0.29	HC1	5.00E-04
PE(34:3)	714.5058	ESI(+), [M+H] ⁺	2	3.62E+0 5 ± 9.72E+0 4	8.84E+0 5 ± 8.97E+0 4	1.44	OxWR	5.62E-04
CL(36:2)(38:2)	1503.099 4	ESI(+), [M+NH ₄] ⁺	1,2	1.62E+0 8 ± 1.87E+0 7	6.88E+0 7 ± 1.57E+0 7	1.36	HC1	7.70E-04

^aSee ESM Table S3 for possible alternative IDs