DEISY GUIMARÃES CARNEIRO

INFLUÊNCIA DO MECANISMO DE *QUORUM SENSING* NO METABOLISMO DE *Salmonella enterica*

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 26 de julho de 2017.

Tiago Antônio de Oliveira Mendes
(Coorientador)

Uelinton Manoel Pinto
(Coorientador)

Maria Emilene Martino Campos-Galvão

Maria Cristina Dantas Vanetti
(Orientadora)

Aos meus pais, Antônio e Sônia,
ao meu irmão Yan,
pelo incentivo e apoio constante.

DEDICO

"Descobri como é bom chegar quando se tem paciência. E para se chegar, onde quer que seja, aprendi que não é preciso dominar a força, mas a razão. É preciso, antes de mais nada, querer."

Amyr Klink

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BIOGRAFIA

DEISY GUIMARÃES CARNEIRO, filha de Antônio Divino Carneiro Filho e Sônia Moreira Guimarães, nasceu em Viçosa - MG, em 09 de dezembro de 1992.

Em janeiro de 2015, graduou-se em Ciências Biológicas pela Universidade Federal de Viçosa.

De junho a julho do mesmo ano, atuou como professora da Educação Básica na Escola Estadual Imaculada Conceição em Porto Firme - MG.

Em agosto de 2015, ingressou no Programa de Pós-Graduação em Microbiologia Agrícola, na área de Microbiologia de Alimentos.

SUMÁRIO

RESUMO

CARNEIRO, Deisy Guimarães, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Influência do mecanismo de *quorum sensing* no metabolismo de *Salmonella enterica*.** Orientadora: Maria Cristina Dantas Vanetti. Coorientadores: Tiago Antônio de Oliveira Mendes e Uelinton Manoel Pinto.

Salmonella é um dos principais patógenos que causam infecções relacionadas ao consumo de alimentos contaminados. *Salmonella enterica* sorotipo Enteritidis (*Salmonella* Enteritidis) é mais comumente associado a surtos clínicos em vários países, incluindo no Brasil. Por meio do mecanismo de *quorum sensing* (*QS*), esse patógeno é capaz de coordenar seu perfil de expressão gênica com a densidade populacional. O mecanismo de *QS* mediado pelo autoindutor-1 (AI-1) acil homoserina lactona (AHL) em *Salmonella* é incompleto, uma vez que não há síntese dessa molécula. Entretanto, esse patógeno é capaz de detectar AHLs produzidas por outras espécies e regular funções celulares. Neste trabalho, foi avaliada a influência de n-dodecanoilhomoserina lactona (C12-HSL) no consumo de glicose e no metabolismo de *Salmonella* Enteritidis PT4 578 cultivada em condições anaeróbias. A análise do sobrenadante do meio de cultura de *Salmonella* por cromatografia líquida de alto desempenho (HPLC) revelou que a absorção de glicose após 4 e 6 h de cultivo na presença de 50 nmol L^{-1} de C12-HSL foi 26 e 29% menor, respectivamente, do que o constatado na ausência de C12-HSL. Este AI-1 também influenciou na abundância de alguns metabólitos intracelulares, analisados em cromatógrafo gasoso acoplado a espectrômetro de massas (GC-MS). Os metabólitos detectados estão relacionados com as vias de metabolismo de glicerolipídios, purinas, aminoácidos e biossíntese de aminoacil-tRNA. A análise do sobrenadante por cromatografia líquida de ultra desempenho acoplada a um sistema de espectrômetro de massa do tipo triploquadrupolo (UPLC-QqQ /MS) revelou que houve aumento de 1,64 vezes na concentração de C12-HSL até 7 h no meio de cultura em que essa molécula foi adicionada. Os dados obtidos demonstraram a influência de C12-HSL exógena no metabolismo de *Salmonella* Enteritidis PT4 578 e, além disso, evidenciaram uma possível síntese de C12-HSL por este patógeno. Por se tratar de resultado ainda não observado anteriormente, esta informação deverá ser confirmada por análises futuras.

ABSTRACT

CARNEIRO, Deisy Guimarães, M.Sc., Universidade Federal de Viçosa, July, 2017. **Influence of the quorum sensing mechanism in the metabolism of *Salmonella enterica*.** Adviser: Maria Cristina Dantas Vanetti. Co-advisers: Tiago Antônio de Oliveira Mendes and Uelinton Manoel Pinto.

Salmonella is one of the major pathogens that cause infections related to the consumption of contaminated food. *Salmonella enterica* serotype Enteritidis (*Salmonella* Enteritidis) is most commonly associated with clinical outbreaks in several countries, including Brazil. By means of the quorum sensing (QS) mechanism, this pathogen is able to coordinate its gene expression profile with the population density. The mechanism of QS mediated by the autoinducer-1 (AI-1) acyl homoserine lactone (AHL) in *Salmonella* is incomplete as there is no synthesis of this molecule. However, this pathogen is capable of detecting AHLs produced by other species and regulating cellular functions. In this work, the influence of n-dodecanoilhomoserine lactone (C12-HSL) on glucose consumption and the metabolism of *Salmonella* Enteritidis PT4 578 cultured under anaerobic conditions was evaluated. Supernatant analysis of the *Salmonella* culture medium by high performance liquid chromatography (HPLC) showed that glucose uptake after 4 and 6 h of cultivation in the presence of 50 nmol L^{-1} C12-HSL was 26 and 29% lower, respectively, than that found in the absence of C12-HSL. This AI-1 also influenced the abundance of some intracellular metabolites, analyzed in gas chromatograph coupled to mass spectrometer (GC-MS). The metabolites detected are related to glycerolipid, purine, amino acid and aminoacyl-tRNA biosynthesis pathways. Analysis of the supernatant by ultra-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer system (UPLC-QqQ / MS) revealed that there was a 1.64-fold increase in C12-HSL concentration up to 7 h of cultivation in the culture medium in that this molecule was added. The data obtained demonstrated the influence of exogenous C12-HSL on the metabolism of *Salmonella* Enteritidis PT4 578 and, in addition, evidenced a possible synthesis of C12-HSL by this pathogen. As this result was not previously observed, this information should be confirmed by future analyzes.

1. REVISÃO DE LITERATURA

1.1. *Salmonella*

Salmonella corresponde ao gênero de bacilos gram-negativos, neutrófilos, anaeróbios facultativos que pertencem à família Enterobacteriaceae; são quimiorganotróficos podem metabolizar nutrientes tanto pelas vias respiratórias quanto fermentativas e tem temperatura ótima de crescimento a 37 °C. Bastante diverso, o gênero atualmente divide-se em duas espécies *S. bongori* e *S. enterica*, sendo esta subdivida em seis subespécies que incluem 2.659 sorotipos (ISSENHUTH-JEANJEAN et al., 2014). *Salmonella* coloniza naturalmente o trato gastrointestinal de humanos e animais e, portanto, é um patógeno facultativo, adquirido pela ingestão de água e alimentos contaminados, principalmente carne de frango e ovos (ANDINO; HANNING, 2015). *Salmonella* pode causar diferentes infecções em humanos, que variam de gastroenterite autolimitada (colite) a febre entérica e infecção sistêmica (bacteremia e sepse) (COBURN; GRASSL; FINLAY, 2007; FABREGA; VILA, 2013; SÁNCHEZ-VARGAS; ABU-EL-HAIJA; GÓMEZ-DUARTE, 2011). A severidade da infecção depende do sorotipo envolvido, virulência, dose infecciosa e imunidade do hospedeiro (CASTILLO et al., 2012).

Anualmente, estima-se que *Salmonella* cause 93,8 milhões de doenças de origem alimentar e 155 mil mortes no mundo (ENG et al., 2015). Em 2013, os custos anuais associados a infecções causadas por *Salmonella* foram estimados em US$ 3,7 bilhões nos Estados Unidos (USDA, 2014). No Brasil, de acordo com a Secretaria de Vigilância à Saúde no período de 2000 a 2015, *Salmonella* foi responsável por 14,4% surtos relatados de doenças causadas pela ingestão de alimentos contaminados (BRASIL, 2015). Das amostras de alimentos envolvidas em surtos no Brasil, *Salmonella* sorotipo Enteritidis PT4 é mais frequentemente encontrado (DOS SANTOS et al., 2003; KOTTWITZ et al., 2010; NUNES et al., 2003).

Uma das características fundamentais para a patogenicidade de *Salmonella* é a capacidade de ajuste do metabolismo para se adaptar as condições adversas encontradas durante a infecção, bem como resistir às respostas imunes do hospedeiro (DANDEKAR et al., 2015; RYCHLIK;

BARROW, 2005). A invasão, a sobrevivência e a replicação são mediadas pela expressão de genes de virulência que, em sua maioria, são agrupados em regiões do DNA denominadas de Ilhas de Patogenicidade (PAIs ou SPIs), provavelmente adquiridas pelo mecanismo de transferência horizontal de genes (KAUR; JAIN, 2012). Mais de 23 SPIs já foram identificadas, sendo SPI-1 e SPI-2 altamente conservadas nos sorotipos de *S. enterica* (FOOKES et al., 2011; NIETO et al., 2016). Além disso, a capacidade de *Salmonella* em aderir e, posteriormente, formar biofilmes em células epiteliais, contribuem para sua resistência e persistência no hospedeiro, uma vez que essa organização celular oferece proteção contra as respostas imunes (STEENACKERS et al., 2012). Por fim, outro fator que contribui para o sucesso de *Salmonella* como patógeno é a capacidade de se comunicar por meio de sinais químicos e regular, de maneira coordenada, a expressão de genes alvos pelo mecanismo de *quorum sensing* (HUGHES; SPERANDIO, 2008; SPERANDIO; KENDALL, 2014).

1.2. *Quorum sensing* (*QS*)

As bactérias em uma comunidade são capazes de interagir e regular, de maneira coordenada, sua resposta a alterações ambientais por meio do sofisticado mecanismo de comunicação celular denominado *quorum sensing* (*QS*) (BASSLER; LOSICK, 2006; FUQUA; WINANS; GREENBERG, 1994; TURAN et al., 2017). A comunicação por *QS* baseia-se na produção de moléculas sinais denominadas autoindutores (AIs) que se difundem para o ambiente, se acumulam e, ao atingir uma concentração limiar, se ligam a receptores celulares e regulam a expressão de genes alvos (HENSE; SCHUSTER, 2015; KELLER; SURETTE, 2006). Essa comunicação química pode ocorrer entre bactérias que pertencem à mesma espécie ou espécies diferentes, como também, entre reinos distintos, envolvendo a bactéria e o seu hospedeiro (HUGHES; SPERANDIO, 2008).

Um grande número de moléculas sinalizadoras ou autoindutoras (AI) é empregado na comunicação por *QS* e, atualmente, existem quatro classes bem definidas:

Acil-homoserina lactonas (AHLs): classe mais comum de AI em gram-negativas, também conhecida como AI-1, são moléculas lipídicas neutras, formadas por um anel de homoserina-lactona com uma cadeia acil, cujo comprimento pode variar de quatro a 18 carbonos e conter modificações (CHURCHILL; CHEN, 2011; YAJIMA, 2014). As AHLs normalmente são produzidas por proteínas homólogas a LuxI, a partir da fração lactona do S–adenosil metionina (SAM) e, na maioria dos casos, a cadeia acil é obtida de intermediários da via de biossíntese de ácidos graxos (PAPENFORT; BASSLER, 2016).

Oligopeptídeos (AIPs): são moléculas de tamanho pequeno utilizadas para a comunicação entre bactérias gram-positivas, são ribossomicamente sintetizadas e algumas vezes passam por modificações pós-traducionais para aumentar a funcionalidade e estabilidade (STURME et al., 2002).

Furanosil borato diéster (AI-2): é considerado um AI universal, utilizado na comunicação intra e interespécie de bactérias gram-negativas e gram-positivas (PEREIRA; THOMPSON; XAVIER, 2013). O AI-2 é produzido a partir de uma S-ribosilhomocisteína (SRH), um produto da via de utilização de S-adenosilmetionina (SAM), que é clivado pela proteína LuxS para formar homocisteína (HC) e o precursor do AI-2 4,5-dihidroxi-2,3-pentadiona (DPD). (DE KEERSMAECKER; SONCK; VANDERLEYDEN, 2006; SKANDAMIS; NYCHAS, 2012).

Autoindutor-3 (AI-3): é um composto aromático aminado produzido por bactérias da microbiota intestinal e por alguns patógenos entéricos como *Escherichia coli* e *Salmonella* (RUL; MONNET, 2015). A estrutura e a síntese do AI-3 ainda são desconhecidas, no entanto, a detecção e a resposta ocorre pelo sistema de dois componentes QseCB, que também responde a hormônios produzidos pelo hospedeiro eucariótico, como epinefrina e norepinefrina e, consequentemente, está envolvido na comunicação entre reinos (HUGHES; SPERANDIO, 2008; LASARRE; FEDERLE, 2013).

O *QS* permite que as bactérias comportem-se como organismos multicelulares e realizem processos que seriam improdutivos e inviáveis se realizados apenas por um indivíduo (BASSLER; LOSICK, 2006). Diversos fenótipos bacterianos já foram descritos como sendo controlados pelo mecanismo de *QS*, entre eles: bioluminescência, competência, biofilme,

virulência, redução da adsorção de bacteriófagos, expressão do sistema CRISPS-cas, metabolismo, síntese de produtos que favorecem apenas a célula produtora e produtos que são excretados e favorecem a população (ALMEIDA et al., 2017; CAMPOS-GALVÃO et al., 2015b; DANDEKAR; CHUGANI; GREENBERG, 2012; HØYLAND-KROGHSBO et al., 2017; HOYLAND-KROGHSBO; MAERKEDAHL; SVENNINGSEN, 2013; JUNG; CHAPMAN; NG, 2015; LI et al., 2002; PARSEK; GREENBERG, 2005; PATTERSON et al., 2016; PONCE et al., 2012; PONCE-ROSSI et al., 2016; RASAMIRAVAKA et al., 2015; SCHUSTER et al., 2013; SCHUSTER; SEXTON; HENSE, 2017; STURBELLE et al., 2015; VERMA; MIYASHIRO, 2013)

1.3. *QS* em *Salmonella*

No gênero *Salmonella*, três sistemas *QS* já foram descritos, mediados pelos AI-1, AI-2 e AI-3 (Figura 1) (SIMÕES; SIMÕES; VIEIRA, 2010; WALTERS; SPERANDIO, 2006).

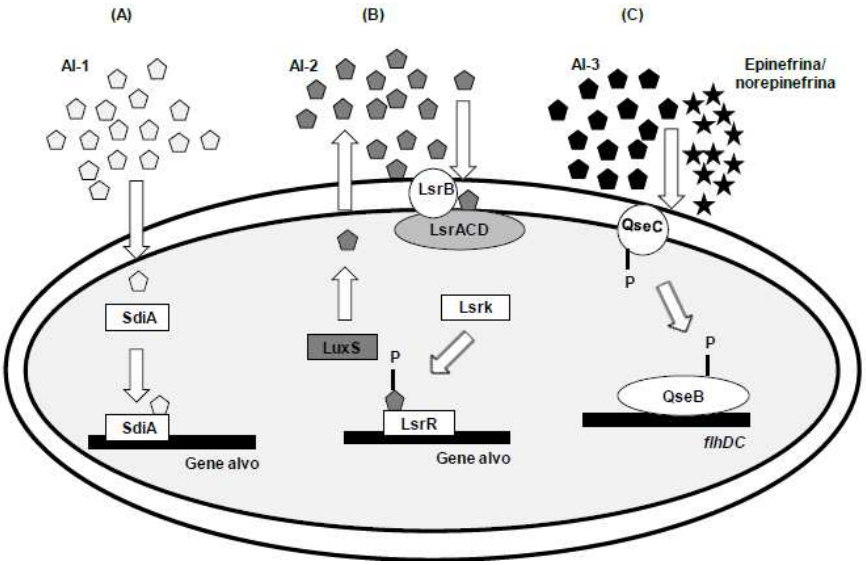

Figura 1. Os três mecanismos de comunicação celular encontrados em *Salmonella*. **(A)** Sistema AI-1/SdiA: A proteína SdiA reconhece e se liga ao AI-1 e regula a transcrição de genes alvo. **(B)** Sistema LuxS/LsrACD: O AI-2 é sintetizado por LuxS, é exportado para o meio extracelular. LsrB reconhece o AI-2 que é internalizado através do transportador LsrACD. No meio intracelular, o AI-2 é fosforilado e se liga LsrR que regula a expressão gênica. **(C)** Sistema AI-3/adrenalina/noradrenalina/QseBC: O AI-3 sintetizado por bactérias ou adrenalina/noradrenalina sintetizados pelo hospedeiro, ao contrário do AI-2, não são internalizados e sim, detectados pelo sensor quinase QseC, que se autofosforila e doa o grupamento fosfato para o regulador de resposta QseB que modula a expressão de genes alvo (adaptada AHMER, 2004; MATA, 2012; WALTERS; SPERANDIO, 2006).

Salmonella não possui o gene *luxI* que codifica a sintase de AHL e, portanto, não sintetiza AI-1, mas é capaz de detectar e responder a AHLs produzidas por outras espécies, pois codifica um homólogo de LuxR, denominado SdiA (BANERJEE; RAY, 2016; MICHAEL et al., 2001). Em *Salmonella* Typhimurium, SdiA regula positivamente a expressão do operon *rck*, que está localizado no plasmídeo de virulência pSLT (SABAG-DAIGLE et al., 2012; SOARES; AHMER, 2011). O operon *rck* contém seis genes, *pefI*, *srgD*, *srgA*, *srgB*, *srgC* e *rck* que codifica a proteína de membrana externa Rck, responsável por um mecanismo de invasão das células epiteliais do hospedeiro independente do sistema de secreção do tipo III (T3SS-1), que é codificado pela SPI-1 (ABED et al., 2014). *Salmonella* Enteritidis também possui o plasmídeo pSLT, porém o mecanismo de regulação da expressão de *rck* por SdiA é diferente do descrito em *Salmonella* Typhimurium (ABED et al., 2014). Além de regular o operon *rck*, SdiA regula também o gene *srgE* (SABAG-DAIGLE et al., 2012; SOARES; AHMER, 2011) que parece ser um gene adquirido por transferência horizontal e que, provavelmente, codifica uma molécula efetora do tipo III (SOARES; AHMER, 2011).

Campos-Galvão et al. (2015a, 2015b) demonstraram que o crescimento de *Salmonella* Enteritidis PT4 não foi influenciado pelo AI-1, mas na presença de N-dodecanoil homoserina lactona (C12-HSL), há aumento na expressão dos genes de virulência *hilA*, *invA* e *invF* pertencentes a PAI-1 e dos genes envolvidos na formação de biofilme *glgC*, *fimF*, *fliF*, após 7 h de cultivo em anaerobiose em caldo triptona de soja (TSB). Nas mesmas condições, as proteínas PrsA, PheT, HtpG, PtsI, TalB, PmgI Eno, PykF e Adi foram positivamente reguladas pela C12-HSL, enquanto OmpA, OmpC, OmpD, GapA, Tsf, RpsB, RplE, RplB foram negativamente reguladas (ALMEIDA et al., 2017). A adição de C12-HSL ao meio de cultivo também promoveu a formação de biofilme mais compacto e maduro em placa de poliestireno após 36 h em anaerobiose, quando comparado com outros AIs de cadeia carbônica variando de seis a 10 carbonos (ALMEIDA et al., 2017; CAMPOS-GALVÃO et al., 2015b). Análises de *docking* molecular feitas por Almeida et al. (2016) demonstraram que C12-HSL tem maior afinidade de ligação à proteína SdiA, quando comparada com AHLs com cadeias carbônicas menores. Esta constatação pode explicar o porquê das alterações nos fenótipos de

Salmonella Enteritidis PT4 578 na presença de C12-HSL. Em *Salmonella* Typhimurium as AHLs produzidas por *Hafnia alvei* não influenciaram na formação de biofilmes em dois substratos abióticos, cupom de aço inoxidável e placa de poliestireno e o resultado foi confirmado com uso de 3OC6-HSL sintética (BLANA; GEORGOMANOU; GIAOURIS, 2017).

O segundo mecanismo de *QS* descrito em *Salmonella* é mediado pelo AI-2, 2R, 4S-2-metil-2,3,3,4-tetrahidroxitetrahidrofurano (R-THMF) sintetizado por LuxS e derivado de 4,5-di-hidroxi-2,3-pentanodiona (DPD) (MILLER et al., 2004). A proteína periplasmática LrsB de *Salmonella* reconhece e se liga ao AI-2, que é internalizado pelo transportador ABC Lsr, composto por LsrA e LsrC. Uma vez internalizado, o AI-2 é fosforilado por LsrK e interage com o repressor transcricional LsrR para aliviar a repressão do operon *lsr* (*lsrACDBFGE*) (BAI; RAI, 2011; PEREIRA; THOMPSON; XAVIER, 2013; TAGA; BASSLER, 2003). Em *E. coli* e *Salmonella,* a síntese, bem como, a importação de AI-2 é estritamente controlada e depende da quantidade de carboidrato no meio e da fase de crescimento; células em fase estacionária não produzem AI-2 e na presença de glicose o AI-2 não é importado, em razão da repressão catabólica exercida pela glicose no operon *lsr* (SPERANDIO; KENDALL, 2014). O estudo da expressão gênica de *Salmonella* Typhimurium selvagem e mutante *luxS* por microarranjo demonstrou que LuxS desempenha função importante na regulação de genes envolvidos no metabolismo e que o sistema *QS* mediado pelo AI-2 está envolvido na expressão diferencial de genes relacionados com a formação de biofilme (JESUDHASAN et al., 2010). Choi et al. (2012) demonstraram que o *QS* mediado pelo AI-2 exerce função importante na regulação da virulência de *Salmonella*, inativando LsrR, que na ausência de AI-2, reduz a expressão de *invF,* gene que regula a expressão de *sicA*, *sigD* e *spoE*, pertencentes a PAI-1.

A sinalização no terceiro mecanismo de comunicação celular em *Salmonella* é mediada por AI aromático bacteriano (AI-3), produzido pela microbiota gastrointestinal normal e pelos hormônios adrenalina/noradrenalina da classe catecolamina produzidos pelo hospedeiro (WALTERS; SPERANDIO, 2006). Neste mecanismo de *QS,* a recepção do sinal é feita pelo sensor quinase que, ao ser fosforilado, ativa a expressão de genes alvos (BAI; RAI, 2011; HUGHES; SPERANDIO, 2008; TURKI et al., 2014). Dois receptores,

QseC e QseE já foram identificados em *E. coli* O157:H7 (EHEC), além disso, estudos mostram que o sistema AI-3 nesse patógeno é responsável pela expressão de genes para formação de lesão em células epiteliais, além de influenciar na ativação do regulon de flagelos responsáveis pela motilidade do tipo *swimming* e síntese de shiga toxina (HUGHES et al., 2009; READING et al., 2009). *Salmonella* Typhimurium codifica ortólogos dos sistemas QseC/QseB e QseE/QseF de *E. coli* e já foi elucidado que QseC influencia na motilidade, além de regular a expressão de SifA, essencial para a sobrevivência intracelular e replicação, enquanto mutantes QseE tem uma redução expressiva na invasão de células HeLa (BEARSON; BEARSON, 2008; MOREIRA; SPERANDIO, 2012; MOREIRA; WEINSHENKER; SPERANDIO, 2010).

Diante da importância do mecanismo de *QS,* especialmente mediado pelo AI-1, na regulação de alguns fenótipos em *Salmonella*, é necessário ampliar as informações a respeito dos processos controlados por essa molécula, a fim de compreender, de uma forma global, seus impactos no comportamento. E, a partir desses conhecimentos, buscar estratégias de controle e inibição desse mecanismo na indústria de alimentos.

1.4 REFERÊNCIAS BIBLIOGRÁFICAS

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Salmonella enterica optimize growth in the presence of acyl homoserine lactone under anaerobic conditions.

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***Salmonella enterica* optimize growth in the presence of acyl homoserine lactone under anaerobic conditions**

Authors: Deisy G. Carneiro[a], Felipe A. de Almeida[a], Thiago A. O. Mendes[b], Nívea M. Vieira[a], Uelinton M. Pinto[c], Maria Cristina D. Vanetti[a]*.

[a] Department of Microbiology, Universidade Federal de Viçosa (UFV), Av. Peter Henry Rolfs, S/N, Viçosa, MG, Brazil;

[b] Department of Biochemistry, Universidade Federal de Viçosa (UFV), Av. Peter Henry Rolfs, S/N, Viçosa, MG, Brazil;

[c] Food Research Center, Department of Food and Experimental Nutrition, Universidade de São Paulo (USP), Av. Professor Lineu Prestes, 580, São Paulo, SP, Brazil.

***** Corresponding author: Universidade Federal de Viçosa (UFV). Av. Peter Henry Rolfs, s/n, Campos Universitário, 36570-000, Viçosa, Minas Gerais, Brazil, Tel: +55 31 3899-2954, Fax: 55 31 3899-2573, E-mail address: mvanetti@ufv.br

Abstract: Acyl homoserine lactones (AHLs) are quorum sensing (QS) signaling molecules that mediate cell-to-cell communication. *Salmonella* does not have a *luxI* homolog which encodes for the AHL synthase therefore; it does not produce these signaling molecules. However, *Salmonella* is able to recognize AHLs produced by other species through SdiA protein which modulates some cellular functions. These cell-to-cell communication molecules can be crucial for pathogenicity of *Salmonella*, playing roles in virulence, biofilm formation, as well as expression of stress-related proteins. We determined the internalization of N-dodecyl-homoserine lactone (C12-HSL), as well evaluated the influence of this signaling molecule on glucose consumption and the metabolic profile of *Salmonella* under anaerobic conditions. Analysis of the supernatant culture in high performance liquid chromatography (HPLC) revealed lower glucose uptake after 4 and 6 h of cultivation in presence of C12-HSL. Subsequent analysis of the supernatant culture by ultra performance liquid chromatography coupled to a triple quadrupole mass spectrometer system (UPLC-QqQ/MS) revealed that there was an increase in C12-HSL concentration for up to 7 h of cultivation in the samples in which this molecule was added. Gas chromatography-mass

spectrometry (GC-MS) based analysis of the intracellular metabolites revealed C12-HSL perturbation in the abundance levels of metabolites related to the pathways of glycerolipids, purines, amino acids and aminoacyl-tRNA biosynthesis. The data obtained suggest an important role of quorum sensing in the adjustment of metabolism for optimization of growth in high population densities. In addition, they serve as a starting point for searching for other AHL synthases other than LuxI in *Salmonella*.
Keywords: quorum sensing, autoinducer, growth stage, metabolic pathway, glucose uptake.

1. Introduction

Salmonella is a facultative intracellular enteric pathogen that infects both humans and animals [1]. Gastrointestinal diseases of infectious origin caused by the ingestion of food contaminated by this pathogen constitute a major public health problem in the world [2]. Annually, it is estimated that *Salmonella* accounts for 93.8 million foodborne illnesses and 155,000 deaths and, among serotypes involved, *Salmonella* Enteritidis has been the most frequently reported [3,4].

The pathogenicity of *Salmonella* is related to the presence of genes that encode virulence factors, usually grouped in regions of the genome called pathogenicity islands [5–7]. In addition, the ability to adjust the metabolism to adapt to the physical conditions and available nutrients found in the host during infection seems to be fundamental for the success of *Salmonella* as a pathogen [8,9].

Quorum sensing is a mechanism of cellular communication used by many bacteria that is based on the production of signaling molecules called auto-inducers (AIs). Usually, AIs diffuse to the environment where they accumulate and, upon reaching a threshold concentration, they bind to intracellular or extracellular receptors regulating the expression of target genes [10–12]. In *Salmonella*, three QS systems have already been described and are mediated by AI-1, AI-2 and AI-3 [13,14].

The first mechanism of QS is mediated by acyl homoserine lactone (AHL) (AI-1), which in gram-negative bacteria, is synthesized by LuxI protein homolog

[15]. *Salmonella* does not contain the *luxI* gene in its genome, thus it does not synthesize AI-1. However, *Salmonella* is able to detect and respond to AHLs produced by other species because it encodes a transcriptional regulator homologous to LuxR proteins called SdiA [16,17]. The second QS mechanism in *Salmonella* is mediated by AI-2, which is synthesized by the LuxS protein, internalized by the transporter Lsr ABC and inside the cell it interacts with the transcriptional repressor LsrR [18–20]. The AI-3 belongs to the third mechanism of QS and it is an amino aromatic compound produced by bacteria of the microbiota, whose detection and response occurs by the two-component system QseCB, which also responds to hormones produced by the host, such as epinephrine and norepinephrine [18,21,22].

Although some studies have investigated the phenotypes regulated by the AI-1 mediated QS system in *Salmonella*, knowledge is still restricted. In *Salmonella* Typhimurium, SdiA positively regulates the expression of the *rck* operon, which is located on the virulence plasmid *p*SLT [23,24]. The presence of different AHLs, with the acyl chain ranging from six to 12 carbons, does not influence the growth of *Salmonella* Enteritidis PT4 578 cultivated in anaerobic TSB medium at 37 °C [25,26]. However, under the same conditions N-dodecanoyl homoserine lactone (C12-HSL) promoted more compact and mature biofilm formation, among the tested AHLs [27, 28]. Moreover, in the presence of C12-HSL, there is an increase in the expression of the virulence genes and genes involved in the formation of biofilm in this pathogen. Alterations in *Salmonella* phenotypes in the presence of C12-AHL can be explained by its higher binding affinity to the SdiA protein, when compared to other AHLs with lower carbon chains as demonstrated by Almeida [29] through molecular docking studies. Cultivation of *Salmonella* Enteritidis PT4 578 in anaerobic TSB medium supplemented with 50 nmol L^{-1} of C12-HSL also led to alterations in protein abundance in early stationary phase of growth [25]. However, despite the evidence of the association between cell density-dependent (AI-1 mediated) gene expression, so far no study has quantified the overall impact of AI-1 on the metabolism of *Salmonella* Enteritidis.

In this work, high performance liquid chromatography (HPLC), liquid chromatography coupled to a triple quadrupole mass spectrometer system (UPLC-QqQ/MS) and gas chromatography-mass spectrometry (GC-MS) were

used to quantitatively investigate the metabolic response of *Salmonella* Enteritidis PT4 578 to C12-HSL under anaerobic conditions.

2. Materials and methods

2.1 Bacterial strain and culture medium

The *S. enterica* serovar Enteritidis PT4 578 (GenBank: 16S ribosomal RNA gene MF066708.1) strain used in this study was isolated from chicken meat and donated by the Oswaldo Cruz Foundation- FIOCRUZ Rio de Janeiro, Brazil. The culture was maintained in Luria Bertani broth (LB) (tryptone 1%, yeast extract 0.5% and NaCl 0.4%) and 20% (v/v) sterile glycerol at -20 °C. Cell culture was performed in Tryptic Soy Broth (TSB; Sigma, USA) medium prepared with CO_2 under O_2 free conditions, dispensed in anaerobic flasks sealed with butyl rubber stopper and autoclaved at 121 °C for 15 min.

2.2 Culture conditions

Before each experiment, cells were cultured two consecutive times in anaerobic bottles containing 10 mL of anaerobic TSB and incubated for 24 h at 37 °C. Then, 0.2 mL were transferred to 20 mL of anaerobic TSB and incubated for 4 h at 37 °C. Then, cells were centrifuged at 12,000 *g* for 10 min at 4 °C (Sorvall, USA), the pellet was washed twice with 1 mL of saline 0.85% and, finally, resuspended in 5 mL of saline 0.85%. The optical density of the cells was standardized at 0.1 at 600 nm (OD_{600nm}) which corresponds to, approximately, 10^7 CFU mL^{-1}, using a spectrophotometer (Thermo Fisher Scientific, Finland) [27]. N-dodecanoyl-DL-homoserine lactone (C12-HSL; PubChem CID:11565426; Fluka, Switzerland) was suspended in acetonitrile (PubChem CID: 6342; Merck, Germany) at a concentration of 10 mM. The choice of C12-HSL was based on studies showing its better effect on biofilm formation by *Salmonella* and due to its greater affinity of SdiA [27–29]. Three flasks containing 100 mL of anaerobic TSB were supplemented with 500 µL of C12-HSL (50 nmol L^{-1}), and other three flasks were supplemented with the same volume of acetonitrile as a control treatment. The acetonitrile concentration was

less than 1% (v/v) in the culture media since, according Michael [17], this concentration does not exhibit effect on the growth and response of *Salmonella* to AHL. The medium was inoculated with 10 mL of the standardized inoculum and incubated at 37 °C for 36 h. Samples were taken at specific time points of 4, 6, 7, 12, 24 and 36 h for analysis.

2.3 Determination of cellular growth, pH and titratable acidity of the medium

Salmonella growth was followed by measuring, at specified times, the absorbance at 600 nm (OD_{600nm}) of the cell suspension using a spectrophotometer (Thermo Fisher Scientific, Finland). A portion of 2.5 mL of culture was also withdrawn and the pH was measured in potentiometer and the titration was performed with 0.1 N sodium hydroxide (NaOH) in the presence of 1% (w/v) phenolphthalein. The results were expressed as $eq.L^{-1}$ of acids produced.

2.4 Analysis of glucose consumption

Glucose consumption was assessed by the quantification of glucose present in the extracellular medium by high performance liquid chromatography (HPLC). At pre-established times, including initial growth time (0 h), 2 mL of the culture were removed, immediately frozen in liquid N_2 and stored at - 80 °C until analysis. The sample was centrifuged 12,000 *g* for 10 min, filtered on 13 mm cellulose nitrate membrane, 22 µm pore (Unifil) and 1 mL of each sample was analyzed in HPLC Dionex Ultimate 3000 (Dionex Corporation, EUA) coupled to a refractive index Shodex RI-101 (Dionex Corporation, USA), maintained at 40 °C. Aminex® HPX-87H (Bio-Rad, USA) ion exchange column 4.6 mm x 300 nm 0.45 µm, maintained at 45 °C and a Micro-Guard Cation H column (Bio-Rad, USA) was used [30]. The mobile phase contained 5 mmol L^{-1} H_2SO_4 (Sigma-Aldrich, USA), the flow was 0.7 mL min^{-1} and the injection of 20 µL. The HPLC was calibrated with the standard glucose curve, prepared at a concentration of 13 mM.

2.5. Sampling and quenching of cell metabolism

A 10 mL aliquot was harvested at each time of sampling and immediately sprayed into 15 mL of 60% methanol/water cold solution for quenching of cellular metabolism [31]. Subsequently, the samples were centrifuged at 12,000 *g* for 10 min at 4 °C. Cell pellets were frozen in liquid N_2 and kept at - 80 ºC until extraction. The supernatant was also frozen in liquid N_2 and maintained at - 80 ºC for quantification of extracellular C12-HSL.

2.5.1 Intracellular metabolite extraction

Intracellular metabolites were extracted from quenched cell pellet using 1.5 mL of cold mixture containing methanol (Sigma-Aldrich, EUA), chloroform (Sigma-Aldrich, USA) and water in the proportions 1: 2.5: 1, following the protocol described by Lisec [32] with modifications. In each sample, 60 µL of ribitol (0.2 $mg.mL^{-1}$ stock in ultra pure water) (Sigma-Aldrich, USA) were added as an internal quantitative standard. The samples were then vortexed and agitated in thermomix (Eppendorf, Germany) for 10 min at 4 °C at 80 *g*. Subsequently, the samples were centrifuged at 11,000 *g* for 10 min at 4 ºC and 1mL of the supernatant was transferred to a new microtube containing 1.5 mL of ultrapure water. The samples were homogenized in vortex and again centrifuged at 14,000 *g* for 15 min at 4 ºC. The upper phase (polar phase) was collected and fractionated into aliquots in 1.5 mL microtubes. Aliquots of 200 µL were evaporated under vacuum in speedvac (Eppendorf, Germany). The samples were then stored at - 80 °C until derivatization.

2.5.2 Chemical derivatization

The samples were derivatized according to the protocol described by Lisec [32]. In summary, 40 µL of methoxyamine hydrochloride (Sigma, USA) at 20 mg mL^{-1} in pure pyridine (Merck, Germany) were added to each tube. The samples were shaken in thermomix (Eppendorf, Germany) for 2 h at 37 °C at 80 *g*. A volume of 70 µL of a solution of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma, USA) with 40 µL mL^{-1} of mix of fatty acid methylesters was added

(FAMEs; Sigma, USA) with standards for the retention time. The samples were shaken in thermomix (Eppendorf, Germany) for 30 min at 37 °C at 80 *g*. Finally, 90 µL of each sample were transferred to glass vials suitable for GC-MS analysis.

2.5.3 Gas chromatography–mass spectrometry (GC-MS) analysis

The samples were analyzed using a system gas chromatography time-of-flight mass spectrometry (GC-TOF-MS), chromatograph Agilent Technologies 7890A spectrometer Leco, TruTOF® HT TOFMS. The capillary column used was a DB-35ms 30 m x 0.32 mm, 0.25 µm (Agilent Technologies). The parameters of injection and analysis were defined according to Lisec [32]. An aliquot of 1 µL of each sample was injected in splitless mode with the injector temperature at 230 °C. The flow of helium gas through the capillary column was adjusted to 2 mL min^{-1}. The temperature remained isothermal for 2 min at 80 °C then, increased by 15 °C per min up to 330 °C and, that temperature was maintained for 6 min.

2.5.4 Data mining, data normalization and data analysis

The chromatograms had their baseline adjusted and the compilation deconvolution algorithm provided by the software ChromaTOF (LECO, Germany) was used for its deconvolution. The deconvoluted spectra were used to assign the peaks using TagSearch software [33] and spectral mass libraries of compounds derived from trimethylsilicon (TMS) obtained from the Max Planck Institute for Plant Molecular Physiology (http://csbdb.mpimp-golm.mpg.de/csbdb). Areas of peak chromatography were verified and normalized by the peak area corresponding to the internal ribitol standard. Principal component analysis (PCA) was performed with the triplicates of each time using software R. The average values of the triplicates were used to construct the heatmap and dendogram, using software R. An analysis of the Pearson correlation coefficient was also made, also using software R and the metabolites with $p > 0.05$ were considered uncorrelated and these were followed for pathway impact analysis using the Metaboanalyst 2.0 software

[34,35], where the data of *Escherichia coli* K12 were used as reference for this analysis.

2.6 AHL quantification

The quenched supernatant was centrifuged at 12,000 *g* for 10 min and filtered through 13 mm Fluoropore PTFE membrane 22 µm (Millipore, USA). The C12-HSL was quantified by the ultra performance liquid chromatography system (UPLC; Infinity 1290, Agilent Technologies, USA) coupled to a triple quadrupole mass spectrometer (QqQ; model 6400, Agilent Technologies) with electrospray ionization (ESI) and atmospheric pressure chemistry ionization (APCI). Chromatographic separations were carried out using an ZORBAX Eclipse Plus C18 column 50 mm x 2.1 mm, 1.8 µm (Agilent Technologies) maintained at 25 °C with a guard column ZORBAX SB-C18, 1.8 µm (Agilent Technologies). The mobile phase for 6 min was composed of 35% acetic acid (Merck, Germany) 0.02% in ultrapure water, pH 4 and 65% acetic acid 0.02% in acetonitrile (Sigma-Aldrich, USA), pH 4 and for 1 min 10% acetic acid 0.02 % in ultrapure water, pH and 90% acetic acid 0.02% in acetonitrile, pH 4. The flow was 0.3 mL min^{-1} and the injection of 10 µL according to Ortori [36] with modifications. The UPLC was calibrated with the standard C12-HSL curve, prepared at the following concentrations: 0, 10, 20, 40, 80, 160 and 200 nM. The obtained equation was area = 6.820 x concentration + 0.9564, with R^2 = 0.9973.

2.7 Statistical analysis

The data of growth, pH, titratable acidity, glucose consumption and C12-HSL quantification were submitted to analysis of variance (ANOVA) and Tukey's test using the Statistical Analysis System and Genetics Software® [37]. A p <0.05 was considered to be statistically significant.

3. Results and Discussion

Salmonella growth, glucose consumption and extracellular C12-HSL concentration (Fig. 1), pH and titratable acidity (Table 1) in the presence of acetonitrile or 50 nmol L^{-1} C12-HSL in anaerobic TSB medium for 36 h was evaluated. As already described by Campos-Galvão [26] and Almeida [25], C12-HSL has no apparent influence on the growth of *Salmonella* Enteritidis PT4 578 (Fig. 1). However, the concentration of glucose in the extracellular medium in the presence of C12-HSL after 4 and 6 h of cultivation was 26 and 29% higher, respectively, indicating a lower consumption of this sugar when compared to the control. Despite the lower glucose consumption in the presence of C12-HSL, OD_{600nm} value indicated a population similar to the control, without C12-HSL. These results suggest that *Salmonella* may have optimized its growth, opting for more energetically favorable metabolic reactions in the presence of the signaling molecule. Studies in the rice pathogen *Burkholderia glumae* using analyzes based on RNAseq showed that strains mutated in the C8-HSL (*tofI*) synthase gene, cognitive receptor (*tofR*) and transcriptional regulator (*qsmR*) significantly increased expression of *ptsI*, a representative gene in the sugar phosphotransferase (PTS) system, compared to the wild type [38]. Moreover, analyzes using nuclear magnetic resonance spectroscopy showed that the transport levels of D-glucose-1-[13C] were significantly higher in the QS mutants than in the wild type. The higher uptake of glucose by the QS mutants in *B. glumae* indicates that the AI-1 QS system in this pathogen acts as an individual metabolic brake when the cells are in high population [38]. Similar behavior was observed in the etiological agent of bulb plague, *Yersinia pestis* in which RNAseq analyzes demonstrated that the *ptsI* gene was downregulated in the wild-type compared to an AHL-null strain during the logarithmic growth phase [39].

After 7 h of cultivation there is no difference in glucose uptake (Fig. 1) and this can be explained by the 1.57-fold increase in the abundance of PtsI protein (phosphoenolpyruvate protein phosphotransferase) in *Salmonella* Enteritidis PT4 578 after 7 h of cultivation in TSB anaerobic medium supplemented with C12-HSL [25]. However, as previous times were not evaluated in the study of Almeida et al. [25], it is a hypothesis.

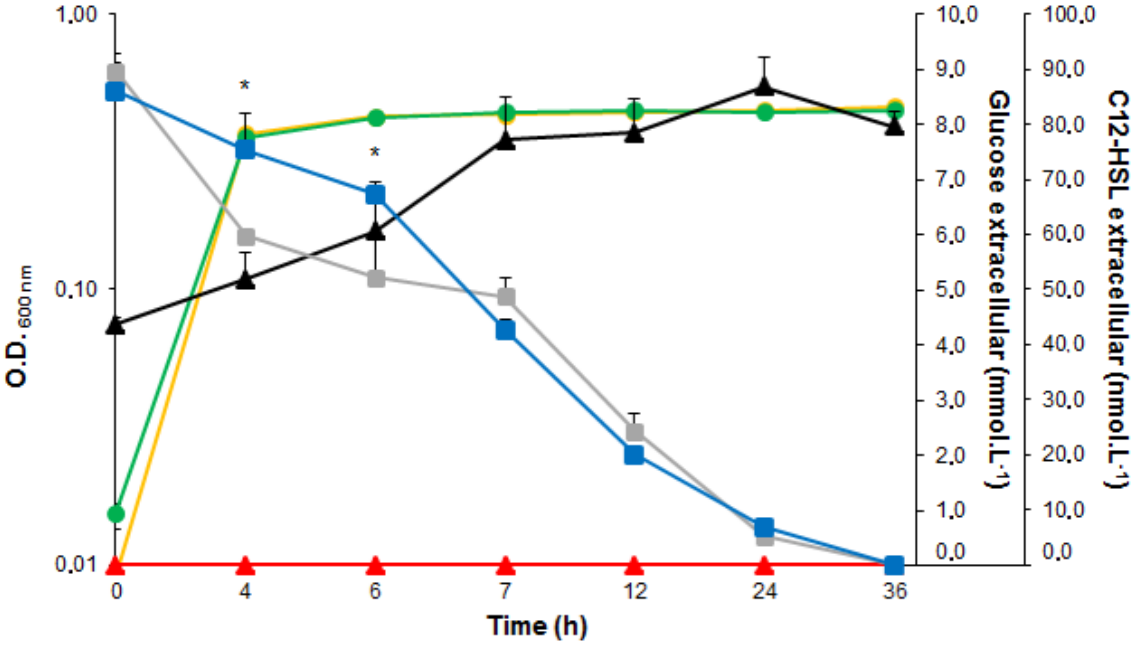

Fig 1. Growth of *Salmonella* Enteritidis PT4 578, glucose and C12-HSL extracellular quantification. *Salmonella* was anaerobically cultivated in TSB at 37 °C for 36 h in the presence of acetonitrile (yellow circle) or 50 nmol L^{-1} of C12-HSL (green circle). Quantification of extracellular glucose in the presence of acetonitrile (gray square) or 50 nmol L^{-1} of C12-HSL (blue square). Quantification of extracellular C12-HSL in the presence of acetonitrile (red triangle) or 50nM of C12-HSL (black triangle). Error bars indicate standard error, and * means significant difference ($p < 0.05$).

Table 1. Evaluation of the pH and titratable acidity of the medium with C12-HSL and control.

Time (h)	pH		Titratable acidity (eq.L^{-1})	
	Control	C12-HSL	Control	C12-HSL
0	6.45^{A}	6.45^{A}	16.06^{E}	16.06^{E}
4	5.81^{bC}	5.90^{aB}	26.10^{D}	26.10^{D}
6	5.49^{bE}	5.56^{aE}	30.12^{C}	30.12^{C}
7	5.35^{F}	5.38^{F}	32.13^{B}	32.13^{B}
12	5.31^{F}	5.28^{G}	34.14^{A}	34.14^{A}
24	5.54^{bD}	5.61^{aD}	30.12^{bC}	32.13^{aB}
36	5.86^{B}	5.83^{C}	30.79^{C}	30.13^{B}

The evaluation was made between control and treatment at specific times and over time. Different lowercase letters on the same line (between control and treatment) and different capital letters in the same column (over time) differs at 5% probability ($p < 0.05$) by Tukey's test. When no lower case letter is shown, there is no statistical difference between control and C12-HSL.

The signaling molecule C12-HSL was not detected in the medium during the growth of *Salmonella* Enteritidis PT4 578 but, when 50 nmol L^{-1} of this molecule was added, the concentration gradually increased 1.64-fold up to 7 h of cultivation and, from that time, the concentration remained virtually equal (Fig 1). It is well reported that in *Salmonella* genome, *luxI* homologous genes are absent and therefore, this pathogen does not synthesize AI-1 [13,24,26,40]. However, no study was performed in the presence of the signal molecule added to the medium or used robust methodologies, such as UPLC-MS, for detection of AHLs from *Salmonella* cultures. One hypothesis to explain this result could be the presence of another AHL synthase sequence in the *Salmonella* genome, different from those related to LuxI. Recently, the sequence of an AHL synthase has been deposited in the GenBank (WP_079809502.1) as belonging to *S. enterica*, and the alignment of this sequence through the BLASTp tool demonstrated that it is more related to bacterial AHLs synthases from the Enterobacteriaceae group (Table S1). However, more studies are needed to obtain confirmation that such a sequence is present in the genome of *Salmonella* Enteritidis PT4 578.

The pH and titratable acidity of the growth medium were evaluated to characterize the occurrence of sublethal acid stress throughout the cultivation time. Both in the absent or in the presence of C12-HSL, there was a gradual reduction of pH and the minimum value reached 5.31 and 5.28 respectively, at 12 h incubation. In the log phase, up to 6 h of cultivation the culture medium without C12-HSL had lower pH (Table 1). This result may be related to the higher glucose consumption observed in this condition (Fig 1). However, the titratable acidity data did not vary in the presence or absence of the signal molecule and therefore, this observation was not confirmed (Table 1).

The intracellular metabolites were evaluated in *Salmonella* cells cultivated in the absence and in the presence of C12-HSL by GC-TOF-MS. Our results show that C12-HSL influenced the levels of metabolites in *Salmonella.* We used Principal component analysis (PCA) in order to understand the data structure and to evaluate the quality of replicates. The score plot generated by submitting intracellular metabolites to PCA is demonstrated in Fig 2. Principal component 1 (PC-1) explained 47.59% of the data variance and PC-2 31.08%. The variation between the replicates is much lower than the variation between

the times, indicating that the metabolic quenching with the 60% methanol/water solution was very suitable for the analysis of *Salmonella* intracellular metabolites. The PCA analysis shows that there is a clear dispersion between the control and C12-HSL samples in the time of 4 h and over the growing time the samples tend to cluster, demonstrating that the changes in the metabolic profile caused by C12-HSL tend to reduce over the cultivation time and, from 24 h, no difference is observed.

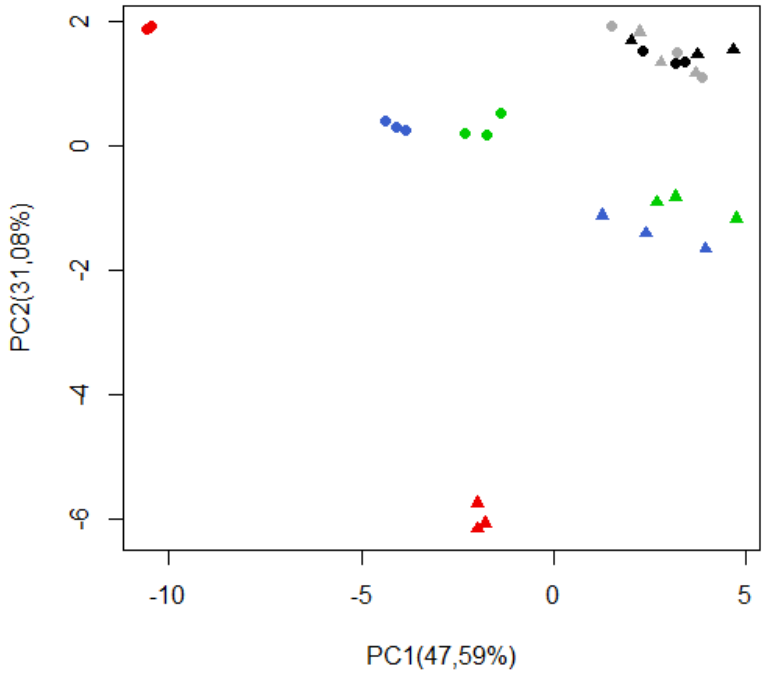

Fig. 2. Principal component analysis (PCA) score plot of metabolites from *Salmonella* Enteritidis PT4 578 anaerobically cultivated in TSB at 37 °C presence of acetonitrile (filled triangle); 50 nmol L^{-1} of C12-HSL (filled circle). The colors represent the times 4 h (red), 6 h (blue), 7 h (green), 24 h (gray) and 36 h (black). Metabolites present 47.59% of the variance explained.

The heatmap (Fig. 3A) shows, in a global way, changes in the relative levels of each metabolite over time. The profile in the time of 4 h, in the presence of C12-HSL, has more variations, and this observation is confirmed by the dendogram resulting from cluster analysis by agglomerative hierarchical methods, presented in Fig. 3B. Interestingly, the addition of C12-HSL resulted in changes in the metabolic profile during the early stages of growth, 4 h (logarithmic growth), 6 h (transition from logarithmic growth to stationary phase) and 7 h (early of stationary phase) and despite the continuous presence of C12-HSL in high concentration (Fig. 1), after these times, the metabolic profile was

not significantly altered. These results corroborate with those observed in *Burkholderia thailandensis* and *Pseudomonas aeruginosa,* when the accumulation of signaling molecules in the medium and the growth phase are determinant for the regulation of population density dependent genes and that most of these genes are regulated at the end of logarithmic phase and beginning of stationary phase [41–43].

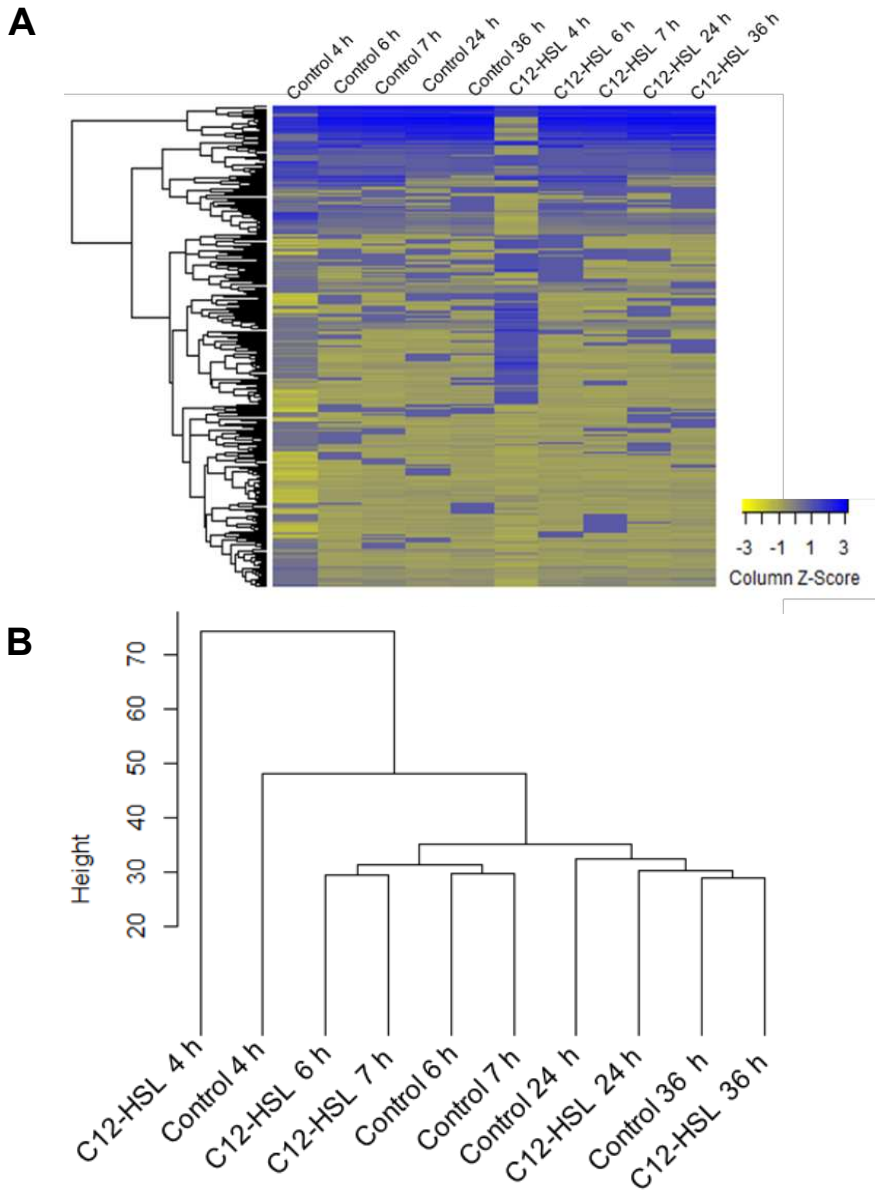

Fig. 3. Heatmap of the relative levels of each metabolite identified by GC-MS over time. Each row corresponds to a unique metabolite, and each column at the mean of the triplicate values. The color scale ranges from yellow (low abundance) to blue (high abundance) (A). Dendogram of the mean values of the triplicates at each time. The height of the arms is proportional to the difference in the abundance profile of the metabolites (B).

Based on uncorrelated metabolites by Pearson's analysis, the metabolic pathway impact revealed the distinct perturbation of C12-HSL in a total of seven pathways (Fig 4). Among these pathways, four are related to amino acid metabolism and the others correspond to the metabolism of purines, glycerolipid and aminoacyl-tRNA biosynthesis (Fig. 5 and Fig. S4-S8).

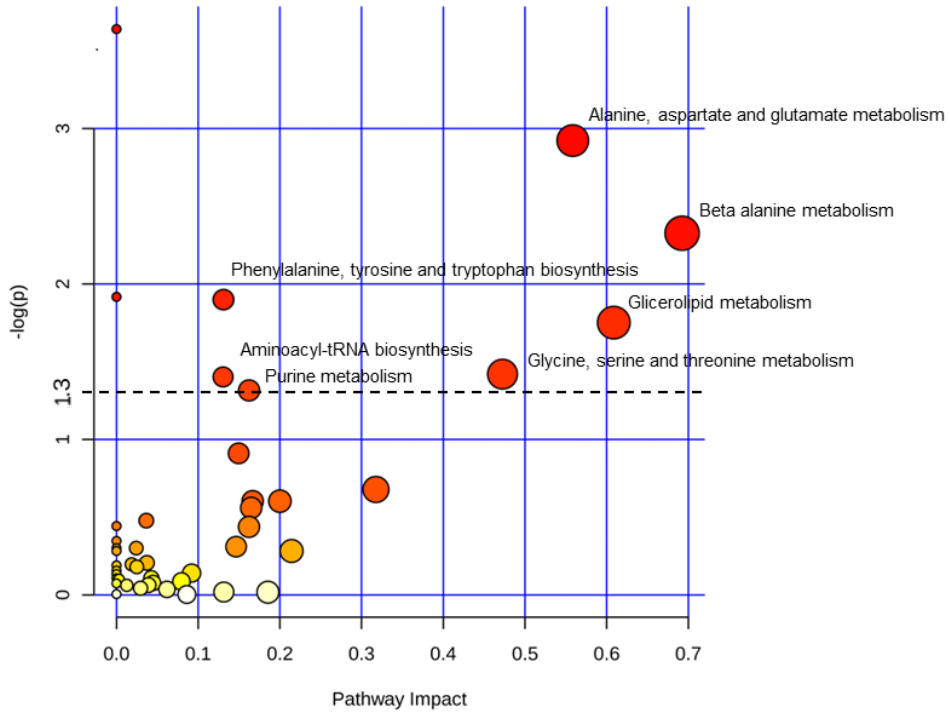

Fig. 4. The pathway impact analysis using MetaboAnalyst 2.0. Metabolic pathways with values –log ≥ 1.3 ($p \geq 0.05$) were considered to be perturbed (above dashed line). The node color is based on its p value and the node radius is determined based on their pathway impact values.

After 4 h cultivation, most of the identified metabolites belonging to the pathways of amino acid metabolism were detected at low levels in the presence of C12-HSL (Fig. 5). Histidine, glutamine, glycine, aspartate, serine, valine, lysine, isoleucine and leucine showed significant abundance decrease in response to C12-HSL (>100-fold) (Fig. S2, Table S2). On the other hand, beta-alanine and threonine were not detected in the control (Fig. S2, Table S3). Since amino acids in the presence of C12-HSL were detected at low levels, the loading of tRNA with its respective amino acids through the aminoacyl biosynthetic pathway was also affected, indicating reduction in protein synthesis (Fig. 5, Table S2). Even after 7 h of cultivation, most amino acids remained less abundant in the presence of C12-HSL (Fig. S6). Analysis of the differential

abundance of proteins separated by electrophoresis in polyacrylamide gel (SDS-PAGE) and identified by mass spectrometer time of flight/time of flight (MALDI-TOF/TOF), demonstrated that in the presence of 50 nmol L^{-1} C12-HSL four proteins related to translation processes, RplB (50S ribosomal protein L2), RplE (50S ribosomal protein L5), RpsB (30S ribosomal protein S2) and the Tsf (elongation factor Ts), were less abundant corroborating previous results [25].

After 4 h of cultivation, the levels of cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), inosine monophosphate (IMP) and adenine were reduced in the presence of C12-HSL (Fig. 5, Table S2). The increase in hypoxanthine and inosine levels may be due to conversion of adenine (Fig. 5). Associated with lower glucose uptake (Fig. 1), these results suggest a reduction in the purine nucleotide synthesis by *de novo* pathway since the this pathway has a high energy cost [44]. The nucleotides play a key role in cellular metabolism, since they are constituents of the genetic material, participate as cofactors in enzymatic reactions, serve as intracellular and extracellular signals, function as phosphate donors and are the main carriers of cellular energy [45]. Nucleotide synthesis can be in two forms: via *de novo* biosynthesis pathway, from amino acids and 5-phosphate ribose or by salvage pathways, a low energy mechanism, initiated from internal and external nucleobases obtained through the hydrolytic degradation of nucleic acids, nucleotides and nucleosides [45–47]. The nucleotide biosynthesis is critical for virulence in *E. coli* and *Salmonella* [48–50]. In *E. coli*, the transcription of the enzymes involved in IMP biosynthesis and the conversion of IMP to AMP and GMP is tightly regulated by the purine repressor (PurR) linked to effector molecules, hypoxanthine or guanine and this mechanism is conserved in *Salmonella* [51,52]. On the other hand, it has already been shown that in *E. coli*, the increase in the intracellular concentration of hypoxanthine is related to the entry in the stationary phase of growth [53,54]. The accumulation of energy-rich metabolites consists of a control strategy to favor cheap metabolic pathways for growth recovery [54]. The ability of QS to anticipate overcrowding and strategies for survival to stationary phase stresses has been reported in three species of the genus *Burkholderia* by Goo et al.[55].

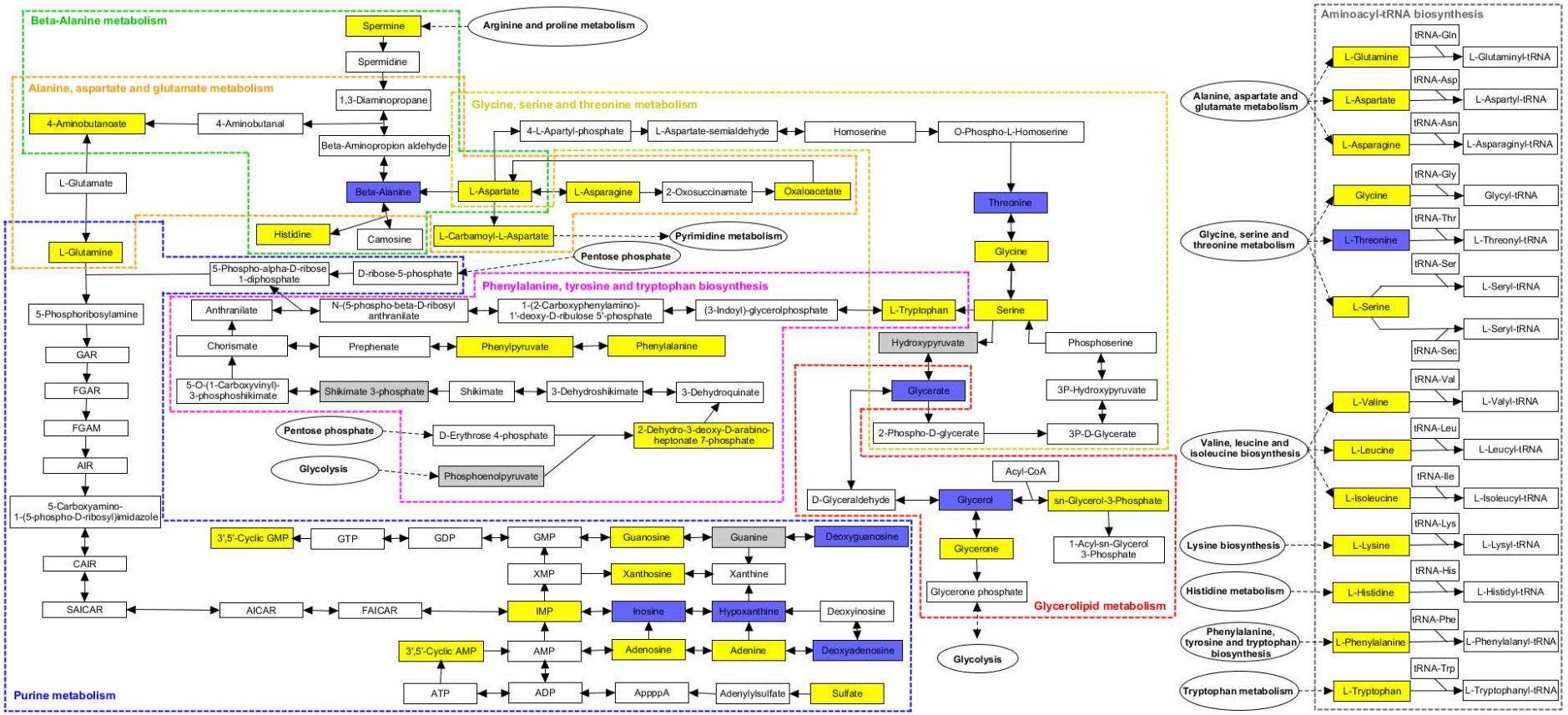

Fig. 5. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL after 4 h of cultivation. The metabolites that had their levels increased in the presence of C12-HSL are shown in blue, those that have been reduced are shown in yellow, and those that did not differ in gray. GAR , 5'-Phosphoribosylglycinamide; FGAR, 5'-Phosphoribosyl-N-formylglycinamide; FGAM, 2-(Formamido)-N1-(5'-phosphoribosyl)acetamidine; AIR, Aminoimidazole ribotide; CAIR, 1-(5-Phospho-D-ribosyl)-5-amino-4-imidazolecarboxylate; SAICAR, 1-(5'-Phosphoribosyl)-5-amino-4-(N-succinocarboxamide)-imidazole; AICAR, 1-(5'-Phosphoribosyl)-5-amino-4-imidazolecarboxamide; FAICAR, 1-(5'-Phosphoribosyl)-5-formamido-4-imidazolecarboxamide; IMP, Inosinic acid; AMP, Adenosine 5'-monophosphate; ADP, Adenosine 5'-diphosphate; ATP, Adenosine 5'-triphosphate; XMP, Xanthosine 5'-phosphate; GMP, Xanthosine 5'-phosphate; GDP, Guanosine 5'-diphosphate; GTP, Guanosine 5'-triphosphate; ApppA, Adenosine 5'-triphosphate 5'-adenosine.

The influence of C12-HSL on the glycerolipid metabolism pathway after 4 h may be due to a homeoviscous adaptation in the *Salmonella* membrane, and, according Zhang [56], this consists of a mechanism that modifies membrane permeability to minimize energy expenditure and optimize growth. Phospholipids are the major constituents of membranes and most of the phospholipids are glycerolipids derived from the acylation of sn-glycerol-3-phosphate (glycerol-3-P) [56]. The influence of C12-HSL on the fatty acid profile of *Salmonella* Enteritidis PT4 578 was verified by Almeida et al. (2017, internal communication) through gas chromatographic analysis using SherlockTM Microbial Identification System (MIS). In this experiment, after 4 h cultivation in the presence of 50 nmol L^{-1} C12-HSL, levels of monounsaturated fatty acids 16:1 ω6c/16:1 ω7c (palmitoleic acid) and 18:1 ω6c/18:1 ω7c (vaccenic acid) increased. These fatty acids have cis-double bonds, which increase the fluidity of the membrane, because the cis double bond introduces a torsion in the acyl chain which occupies a higher molecular volume and is not associated as densely as the unsaturated fatty acids [56,57]. In addition, the main changes in the fatty acid profile demonstrated by Almeida (2017, Internal communication) occurred at the initial growth times, after 4, 6 and 7 h of cultivation, as well as the changes in the metabolic profile observed in this work at the same growing conditions. These results indicate that QS contributes to the development of a more fluid membrane that may be related to lower energy expenditure for nutrient absorption, which may justify lower glucose consumption and even growth.

4. Conclusion

Although some studies have already investigated the mechanism of QS in *Salmonella*, this is the first that focused quantitatively on the response of *Salmonella* Enteritidis to AI-1 under anaerobic conditions. The results show that C12-HSL changes the metabolic profile of *Salmonella* Enteritidis, under anaerobic conditions, reducing the levels of metabolites involved in important metabolic pathways and reducing glucose absorption. However, despite the reduction of metabolism, the growth of *Salmonella* is not affected, suggesting that there is an optimization of the growth in conditions of high population

density. The changes in the metabolic profile accentuated in the initial stages of growth corroborate with the literature data on the importance of the growth phase as well as the concentration of AHL in the regulation of genes by the mechanism of QS. In addition, this study evidences a possible synthesis of AI-1 and provides a new puzzle to be unveiled in *Salmonella*. Given the limited number of studies on the processes regulated by QS, specifically AI-1, in *Salmonella*, this work points out new information on the influence of this signaling molecule on the metabolism of the pathogen most frequently reported in outbreaks involving the consumption of contaminated foods.

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6. Supplementary Figures

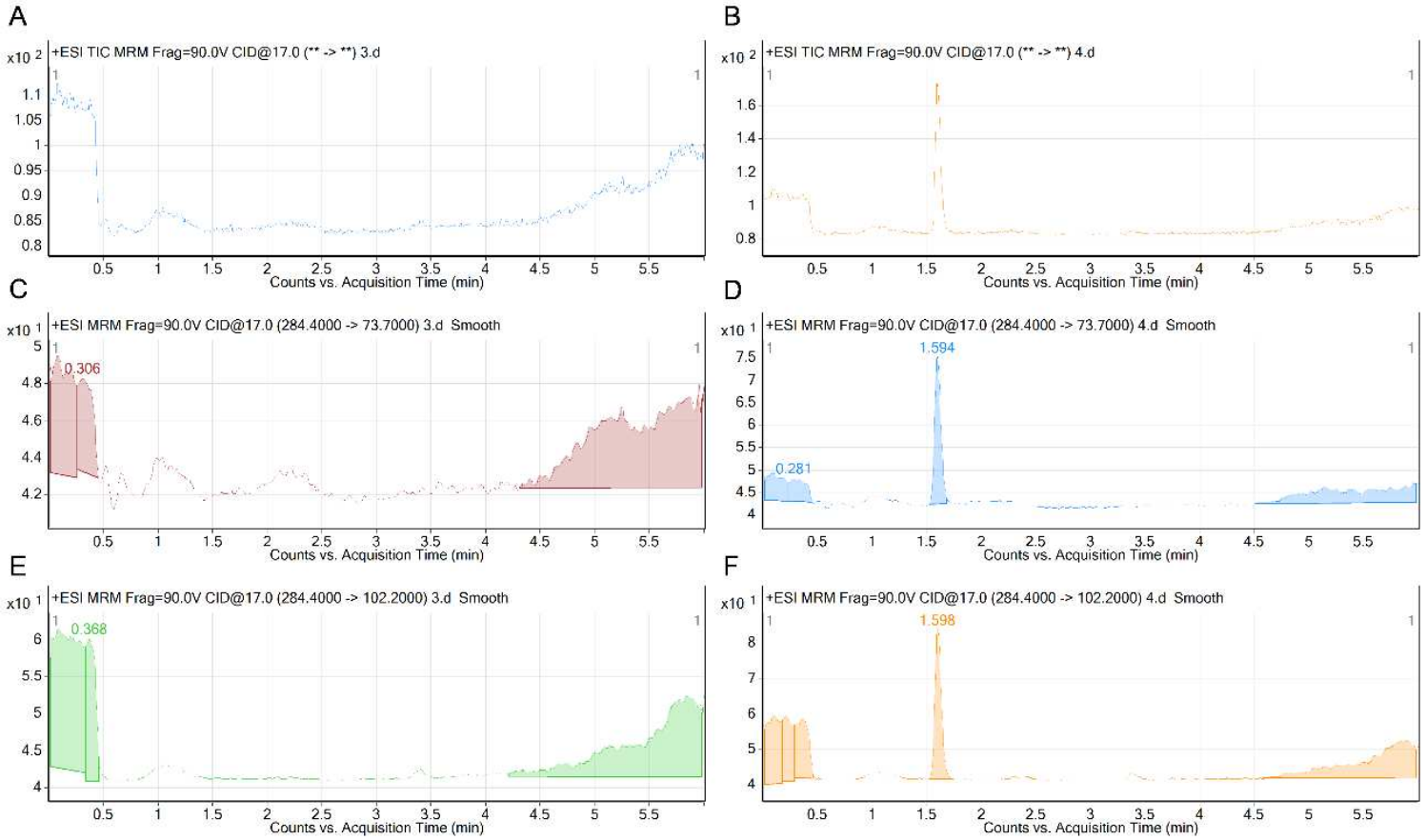

Fig. S1. Ion precursor of C12-HSL control (A) and treatment (B). Product ion (*m/z*) spectrum 73.7000 control (C) and treatment (D). Product ion (m/z) spectrum 102.2000 control (E) and treatment (F). The area of the last scaling was used to calculate the concentration of C12-HSL.

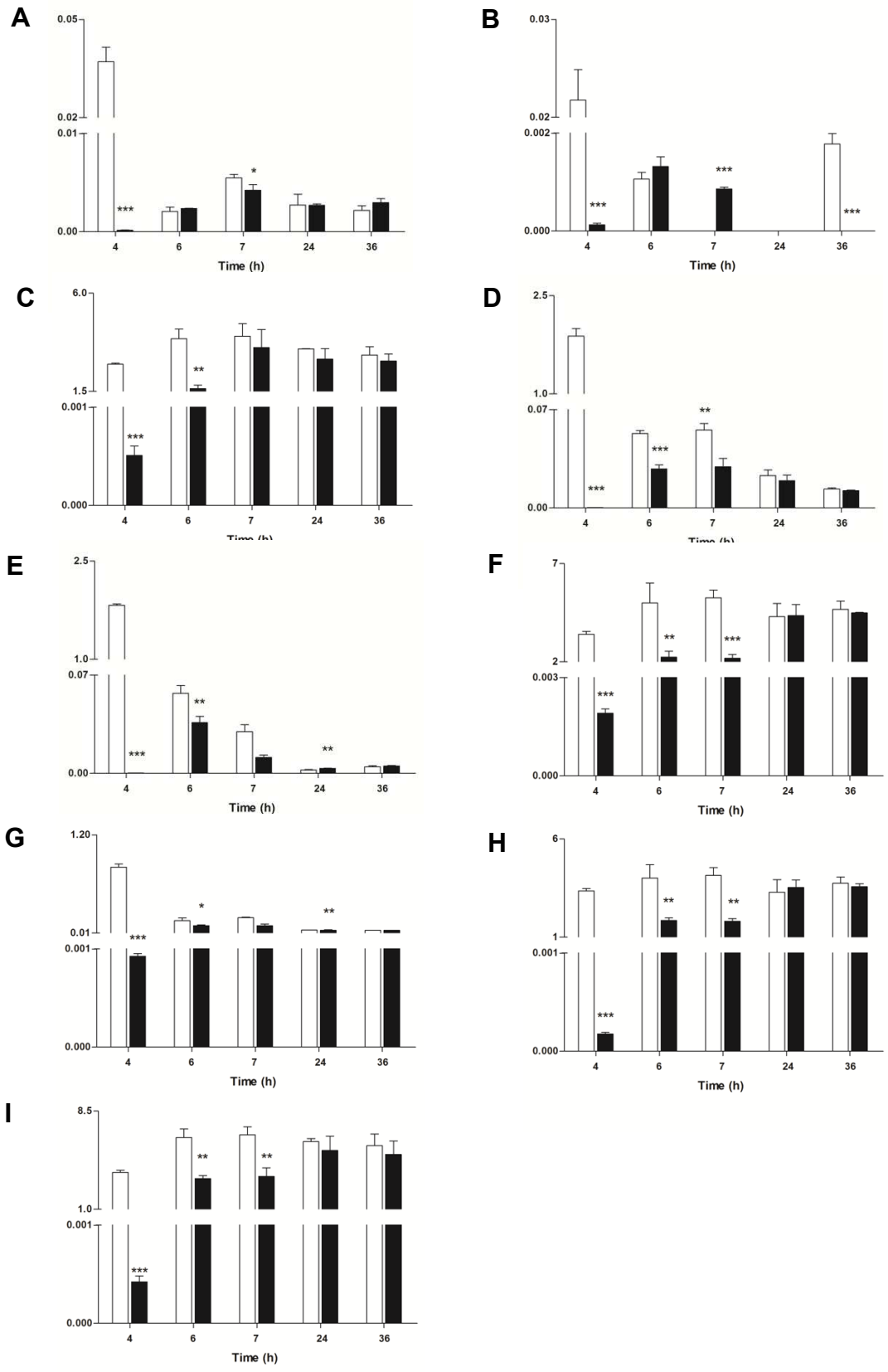

Fig. S2. Metabolites that had their levels reduced in the presence of C12-HSL after 4 h of culture. Treatment (black bars) and control (white bars).(A) histidine, (B) glutamine, (C) glycine, (D) aspartate, (E) serine, (F) alanine, (G) lysine, (H) isoleucine (I) leucine. Values that are significantly different of the control value by Tukey's test are indicated $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

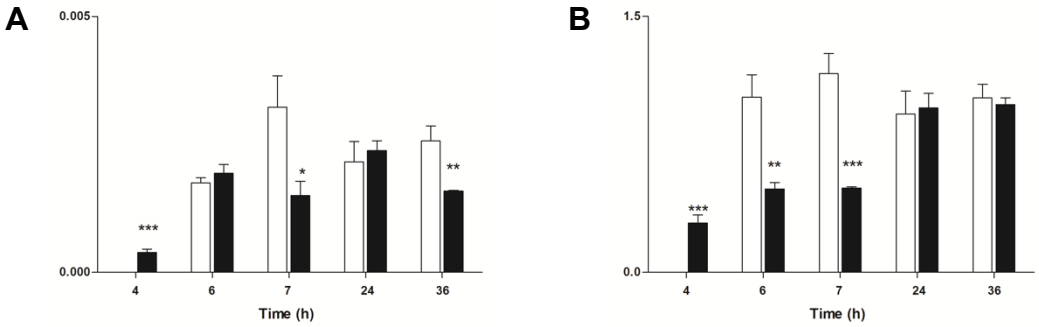

Fig. S3. Metabolites that had their levels increased in the presence of C12-HSL after 4 h of culture. Treatment (black bars) and control (white bars). (A) beta-alanine, (B) threonine. Values that are significantly different of the control value by Tukey's test are indicated $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

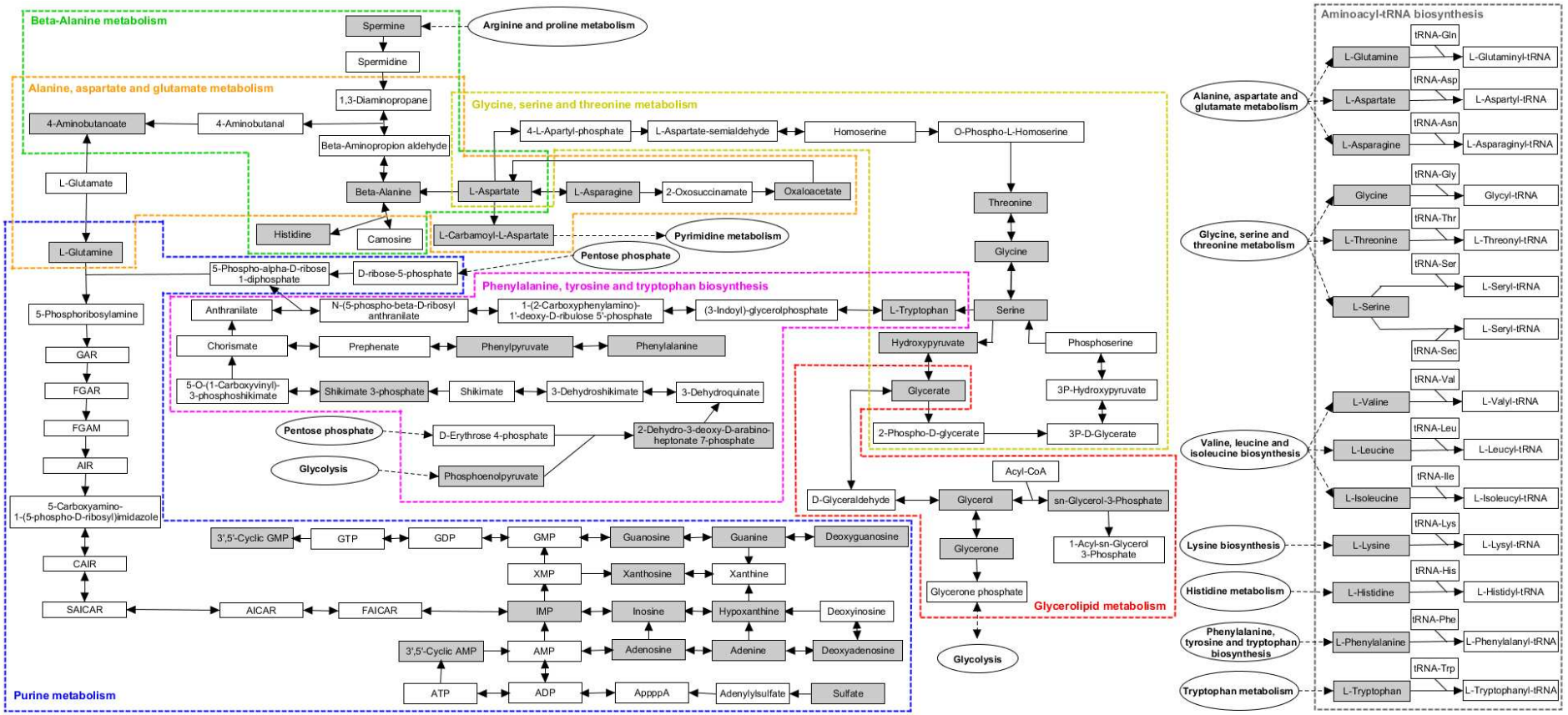

Fig. S4. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL. The metabolites that were identified as differentially expressed in the analyzes are shown in gray.

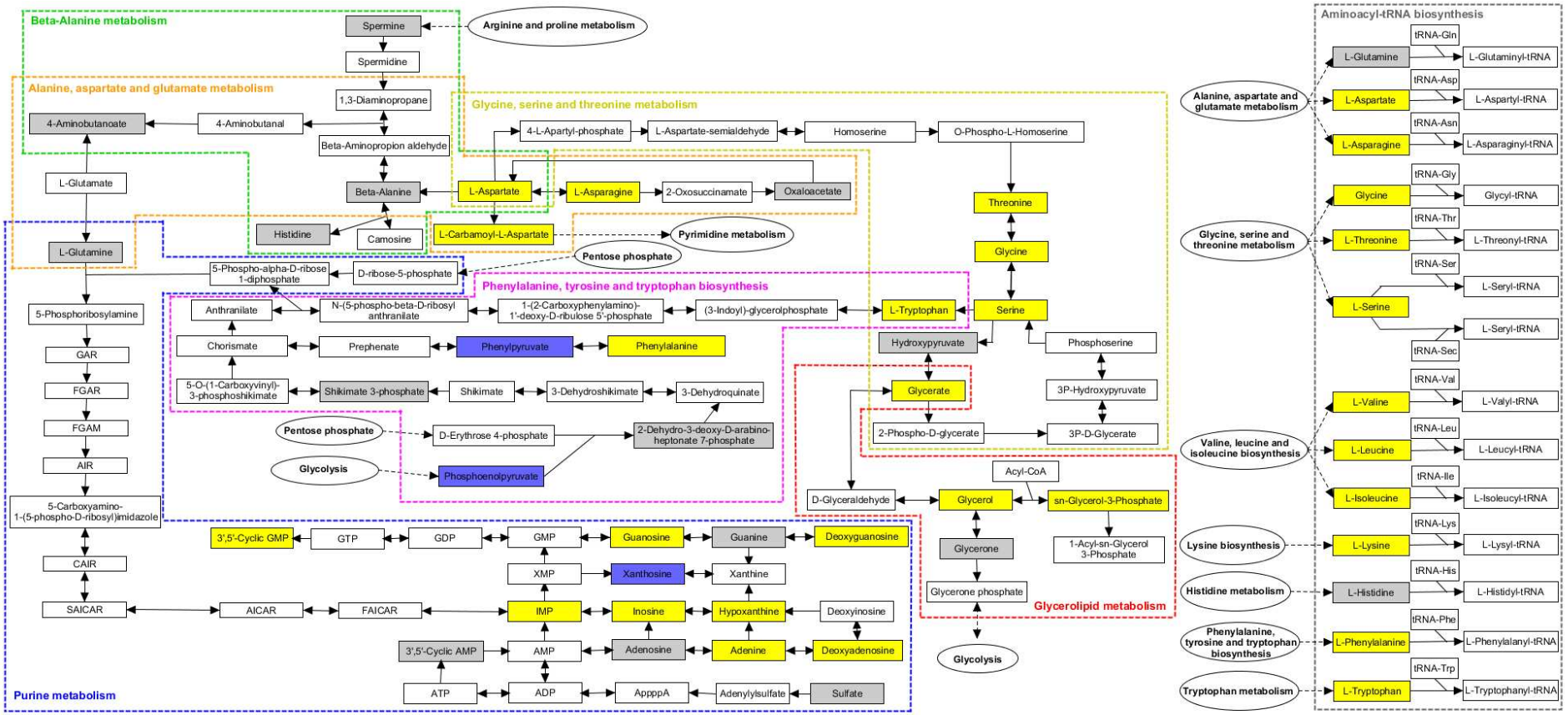

Fig. S5. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL after 6 h of cultivation. The metabolites that had their levels increased in the presence of C12-HSL are shown in blue, those that have been reduced are shown in yellow, and those that did not differ in gray.

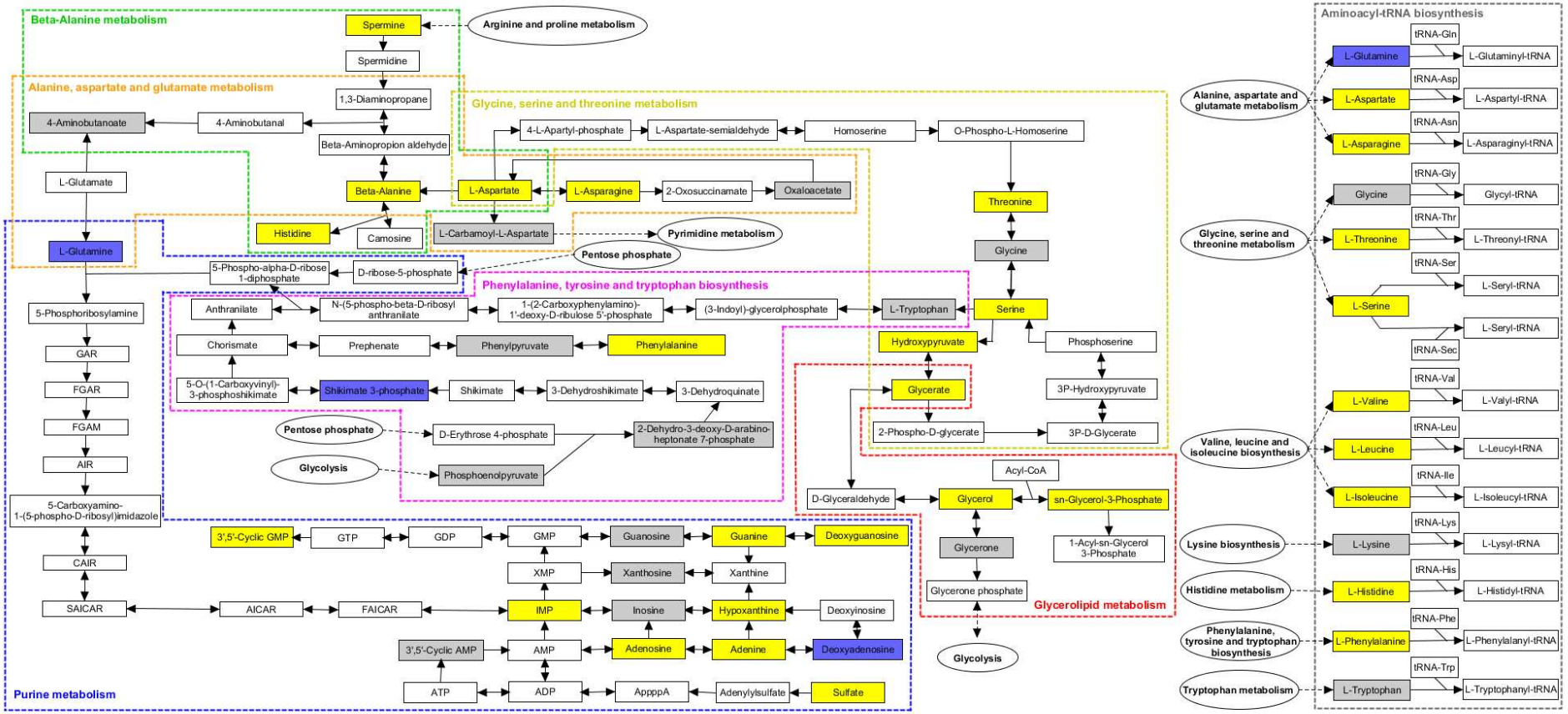

Fig. S6. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL after 7 h of cultivation. The metabolites that had their levels increased in the presence of C12-HSL are shown in blue, those that have been reduced are shown in yellow, and those that did not differ in gray.

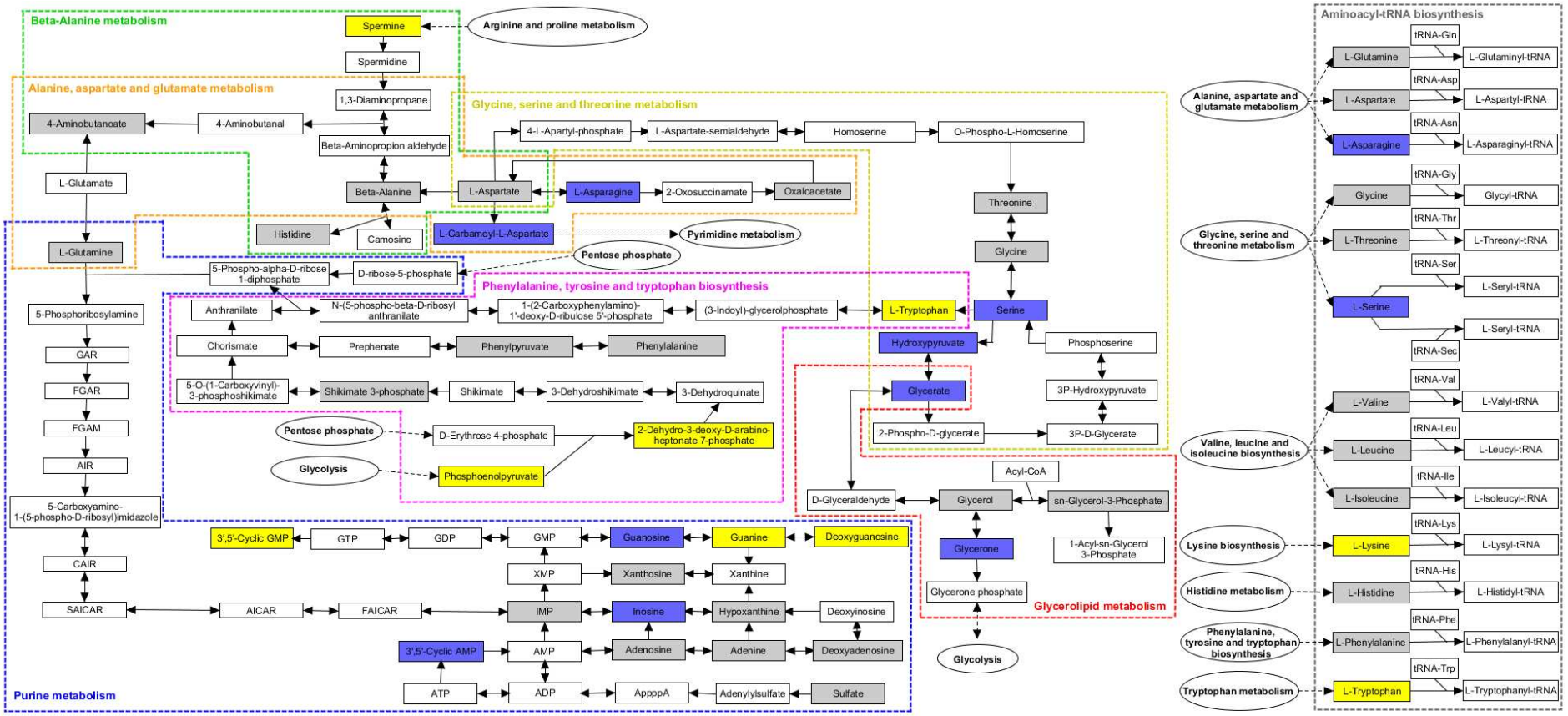

Fig S7. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL after 24 h of cultivation. The metabolites that had their levels increased in the presence of C12-HSL are shown in blue, those that have been reduced are shown in yellow, and those that did not differ in gray.

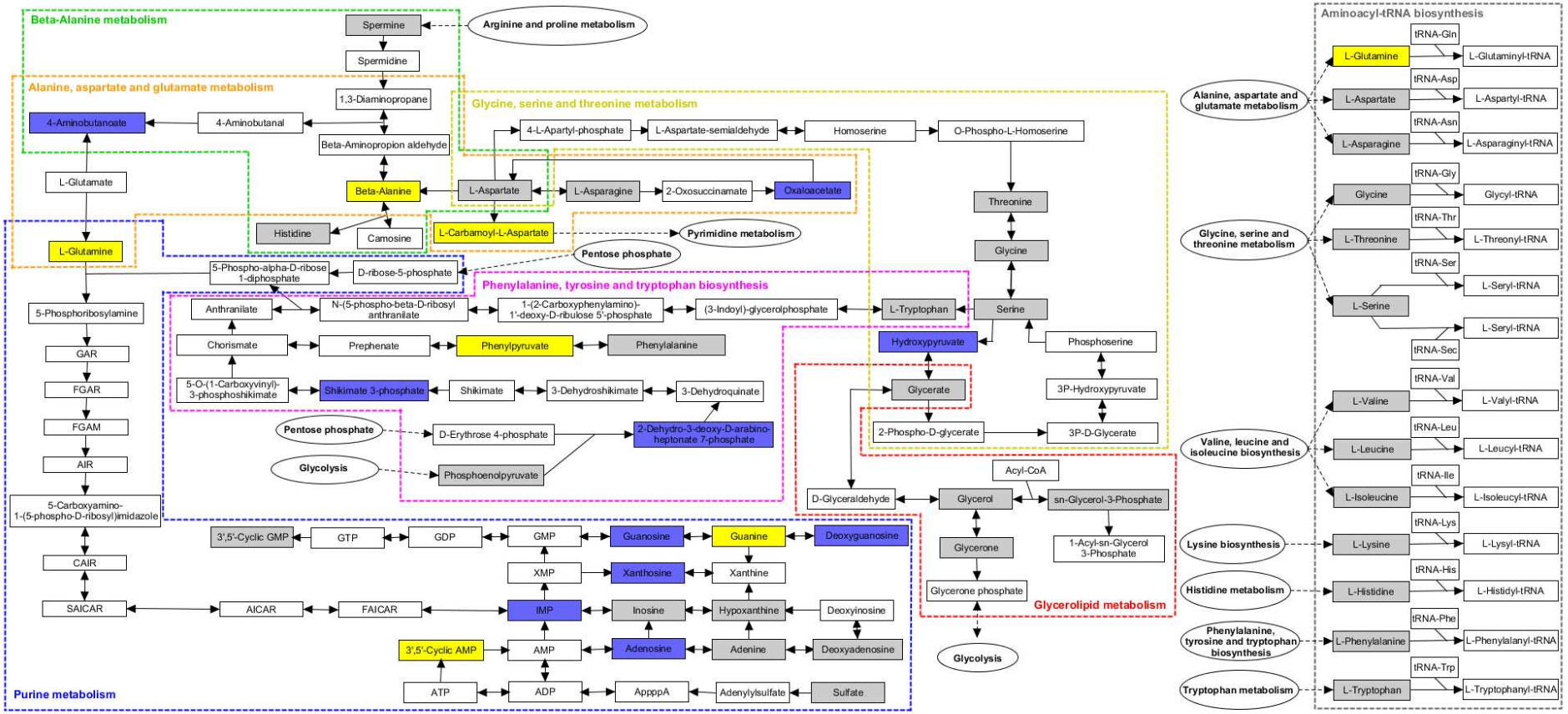

Fig S8. Pathways altered in the presence of 50 nmol L^{-1} C12-HSL after 36 h of cultivation. The metabolites that had their levels increased in the presence of C12-HSL are shown in blue, those that have been reduced are shown in yellow, and those that did not differ in gray.

7. Supplementary Tables

Table S1. Result of alignment of the deposited AHL synthase sequence for *Salmonella enterica* deposited in the GenBank (WP_079809502.1).

Organism	Accession	Description	Maximum score	Total score	Query cover (%)	E-value	Identity (%)
Erwinia gerundensis	WP_067435867.1	Acyl-homoserine-lactone synthase	281	281	89	2.00E-93	65
Brenneria sp. EniD312	WP_009112309.1	Acyl-homoserine-lactone synthase	279	279	89	2.00E-92	65
Serratia marcescens	BAF31199.1	AHL-synthase	276	276	89	1.00E-91	66
Serratia proteamaculans	AGB07458.1	Quorum sensing regulator SprI	276	276	90	1.00E-91	65
Yersinia enterocolitica LC20	AHM76737.1	Autoinducer synthesis protein EsaI	276	276	91	2.00E-91	64
Pantoea sp. OXWO6B1	WP_063879676.1	Acyl-homoserine-lactone synthase	272	272	90	9.00E-90	61
Pantoea allii	ORM87304.1	Acyl-homoserine-lactone synthase	271	271	90	2.00E-89	61
Erwinia sp. ErVv1	WP_067700374.1	Acyl-homoserine-lactone synthase	270	270	92	5.00E-89	61
Serratia ficaria	WP_061799837.1	acyl-homoserine-lactone synthase	269	269	90	1.00E-88	62
Chania multitudinisentens	WP_024912648.1	acyl-homoserine-lactone synthase	269	269	89	1.00E-88	64
Serratia sp. Leaf51	WP_056772484.1	acyl-homoserine-lactone synthase	269	269	90	1.00E-88	63
Pantoea ananatis LMG 20103	ADD77122.1	EanI	268	268	91	4.00E-88	60
Serratia liquefaciens	WP_046374783.1	Acyl-homoserine-lactone synthase	268	268	89	5.00E-88	64
Serratia proteamaculans	AAK76733.1	Quorum sensing regulator SprI	267	267	90	6.00E-88	62
Serratia plymuthica	ACR22886.1	SplI	267	267	90	8.00E-88	62
Pantoea ananatis	WP_028724276.1	Acyl-homoserine-lactone synthase	267	267	90	8.00E-88	60
Erwinia tracheiphila	WP_016192680.1	AHL-synthase EanI	266	266	90	1.00E-87	60
Hafnia alvei	WP_072309736.1	Acyl-homoserine-lactone synthase	266	266	92	1.00E-87	62

Table S2. Variation of the metabolites of the pathways most impacted by C12-HSL.

Time (h)	4		6		7		24		36	
Pathaway / Metabolite	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value
Alanine, aspartate and glutamate metabolism										
L-Aspartate	-12.90	< 0.0001	-0.93	0.0003	-0.92	0.0038	-0.24	0.3273	-0.13	0.0933
L-Glutamine	-7.39	0.0003	0.32	0.1328	>0.00	< 0.0001	-	-	<0.00	0.0001
L-Asparagine	-2.41	< 0.0001	-1.40	0.0004	-1.15	0.0022	0.76	0.0203	-0.14	0.2264
Oxaloacetate	-7.77	< 0.0001	-1.12	0.1481	1.2	0.1181	0.53	0.0788	1.51	0.003
L-Carbamoyl-L-Aspartate	-4.13	< 0.0001	-0.94	< 0.0001	-0.42	0.2235	>0.00	0.0008	-1.02	0.0026
4-Aminobutanoato	-11.39	0.0002	-	-	-	-	-	-	>0.00	< 0.0001
Beta Alanine metabolism										
L-Aspartate	-12.90	< 0.0001	-0.93	0.0003	-0.92	0.0038	-0.24	0.3273	-0.13	0.0933
Spermine	-2.64	0.0023	-0.09	0.3527	-0.44	0.0102	0.00	< 0.0001	0.19	0.0296
Beta-Alanine	>0.00	0.0006	0.15	0.1814	-1.10	0.0115	0.14	0.4499	-0.69	0.0047
L-Histidine	-7.90	0.0001	0.18	0.3246	-0.38	0.0301	-0.02	0.956	0.43	0.0986
4-Aminobutanoato	-11.39	0.0002	-	-	-	-	-	-	>0.00	< 0.0001
Glycine, serine and threonine metabolism										
L-Aspartate	-12.90	< 0.0001	-0.93	0.0003	-0.92	0.0038	-0.24	0.3273	-0.13	0.0933
L-Threonine	>0.00	0.0004	-1.07	0.0024	-1.24	0.0006	0.05	0.7095	-0.06	0.4929
L-Glycine	-12.40	< 0.0001	-1.27	0.0011	-0.20	0.4183	-0.21	0.1603	-0.13	0.4009
Glycerate	>0.00	0.0005	-1.43	0.0055	-1.70	0.0011	0.59	0.0234	0.21	0.1765
Hydroxypyruvate	-0.13	0.1786	0.01	0.9695	-1.14	< 0.0001	0.42	0.0037	0.60	< 0.0001
L-Serine	-13.02	< 0.0001	-0.66	0.0068	-1.38	0.0039	0.58	0.0033	0.16	0.3321
L-Tryptophan	-4.00	< 0.0001	-0.77	< 0.0001	-0.30	0.1606	-0.39	0.0251	0.39	0.1289
Phenylalanine, tyrosine and tryptophan biosynthesis										
Phosphoenolpyruvate	-	-	0.83	0.0017	-	-	<0.00	< 0.0001	-	-
2-Dehydro-3-deoxy-D-arabino-heptonate 7-phosphate	-6.02	< 0.0001	-0.19	0.5491	-	-	<0.00	< 0.0001	0.47	0.021
Shikimate 3-phosphate	-	-	-	-	>0.00	< 0.0001	0.12	0.4054	>0.00	0.0056
Phenylpyruvate	<0.00	0.0005	>0.00	< 0.0001	-0.60	0.1409	-	-	<0.00	< 0.0001
L-Phenylalanine	-4.49	< 0.0001	-0.95	0.001	-0.85	0.0002	0.08	0.7077	0.02	0.8465
L-Tryptophan	-4.00	< 0.0001	-0.77	< 0.0001	-0.30	0.1606	-0.39	0.0251	0.38	0.1289

Table S2. Continuation.

Time (h)	4		6		7		24		36	
Pathaway / Metabolite	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value
Aminoacyl-tRNA biosynthesis										
L-Asparagine	-2.41	< 0.0001	-1.40	0.0004	-1.15	0.0022	0.76	0.0203	-0.14	0.2264
L-Histidine	-7.90	0.0001	0.18	0.3246	-0.39	0.0301	-0.02	0.956	0.43	0.0986
L-Phenylalanine	-4.49	< 0.0001	-0.95	0.001	-0.85	0.0002	0.08	0.7077	0.02	0.8465
L-Glutamine	-7.39	0.0003	0.32	0.1328	>0.00	< 0.0001	-	-	<0.00	0.0001
L-Glycine	-12.40	< 0.0001	-1.27	0.0011	-0.19	0.4183	-0.21	0.1603	-0.13	0.4009
L-Aspartate	-12.90	< 0.0001	-0.93	0.0003	-0.92	0.0038	-0.24	0.3273	-0.13	0.0933
L-Serine	-13.02	< 0.0001	-0.66	0.0068	-1.38	0.0039	0.58	0.0033	0.16	0.3321
L-Valine	-10.79	< 0.0001	-1.16	0.0108	-1.27	0.0003	0.02	0.9197	-0.06	0.493
L-Lysine	-9.77	< 0.0001	-0.67	0.0357	-0.97	0.0016	-0.03	0.7236	-0.01	0.809
L-Isoleucine	-14.23	< 0.0001	-1.12	0.0057	-1.19	0.0006	0.10	0.6135	-0,07	0.4518
L-Leucine	-13.14	< 0.0001	-0.96	0.0014	-0.92	0.0037	-0.16	0.3656	-0.17	0.4454
L-Threonine	>0.00	0.0004	-1.70	0.0024	-1.24	0.0006	0.05	0.7095	-0.06	0.4929
L-Tryptophan	-4.00	< 0.0001	-0.77	< 0.0001	-0.30	0.1606	-0.39	0.0251	0.39	0.1289
Purine metabolism										
Sulfate	-1.59	< 0.0001	-0.32	0.1205	-1.05	0.0049	0.09	0.5141	0.01	0.8413
L-Glutamine	-7.39	0.0003	0.32	0.1328	>0.00	< 0.0001	-	-	<0.00	0.0001
3',5'-cyclic AMP	-5.44	0.0002	-0.42	0.1582	-	-	>0.00	0.001	<0.00	0.0004
3',5'-Cyclic GMP	<0.00	< 0.0001	-0.36	0.0037	-0.77	0.0086	<0.00	0.0025	-	-
Deoxyguanosine	>0.00	0.0007	<0.00	< 0.0001	-0.66	0.0095	<0.00	0.0002	>0.00	0.0006
Guanine	-	-	-0.27	0.1291	<0.00	0.0081	<0.00	< 0.0001	<0.00	< 0.0001
Inosinic acid	-1.30	0.0009	-0.52	0.0203	<0.00	< 0.0001	-0.35	0.0706	0.35	0.024
Guanosine	-7.02	< 0.0001	<0.00	< 0.0001	0.07	0.6992	>0.00	< 0.0001	>0.00	< 0.0001
Adenosine	<0.00	< 0.0001	0.34	0.0615	<0.00	0.0018	0.05	0.7905	>0.00	< 0.0001
Adenine	-11.35	0.0001	-0.87	0.0097	-0.77	0.0024	0.15	0.3968	-0.07	0.7115
Inosine	>0.00	0.0005	<0.00	0.0013	-0.31	0.2823	>0.00	< 0.0001	-0.60	0.0749
Deoxyadenosine	>0.00	0.0062	-0.39	0.0083	>0.00	< 0.0001	-0.56	0.3428	0.18	0.4183
Hypoxanthine	1.07	0.0059	-1.18	0.0002	-0.92	0.0017	0.09	0.9451	0.09	0.4475
Xanthosine	<0.00	0.0009	>0.00	< 0.0001	-0.23	0.2466	0.06	0.7056	>0.00	< 0.0001

Table S2. Continuation.

Time (h)	4		6		7		24		36	
Pathaway / Metabolite	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value	$log_2^{T/C}$	*p* value
Glycerolipid metabolism										
Glycerol	>0.00	0.0012	-1.29	0.0004	-1.32	0.0013	-0.13	0.3174	-0.04	0.7049
Glycerate	>0.00	0.0005	-1.43	0.0055	-1.70	0.0011	0.60	0.0234	0.21	0.1765
sn-Glycerol-3-Phosphate	-6.55	< 0.0001	-1.15	0.0012	-0.95	0.0042	0.05	0.68	0.21	0.3784
Glycerone	-1.20	0.001	-0.05	0.847	0.16	0.283	>0.00	< 0.0001	-0.29	0.2382

Log_2 of the treatment (T)/control (C) ratio= $log_2^{T/C}$; Metabolite not detected in the control = >0.00; Metabolite not detected in the treatment = <0.00.