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Article

Human pleural fluid and human serum albumin modulate the behavior of a hypervirulent and multidrug-resistant (MDR) *Acinetobacter baumannii* representative strain

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Abstract: *Acinetobacter baumannii* is a nosocomial pathogen capable of causing serious infections associated with high rates of morbidity and mortality. Due to its antimicrobial drug resistance profile, *A. baumannii* is categorized as an urgent priority pathogen by the Centers for Disease Control and Prevention in the United States and priority group 1 critical microorganism by the World Health Organization. Understanding how *A. baumannii* adapts to different host environments may provide critical insights into strategically targeting this pathogen with novel antimicrobial and biological therapeutics. Exposure to human fluids was previously shown to alter the gene expression profile of a highly drug susceptible *A. baumannii* strain A118 leading to persistence and survival of this pathogen. Herein, we explore the impact of human pleural fluid (HPF) and human serum albumin (HSA) on the gene expression profile of a highly multi-drug resistant strain of *A. baumannii* AB5075. Differential expression was observed for ~30 genes, whose products are involved in quorum sensing, quorum quenching, iron acquisition, fatty acid metabolism, biofilm formation, secretion systems and type IV pilus formation. Phenotypic and further transcriptomic analysis using quantitative RT-PCR confirmed RNA-seq data and pointed out a distinctive role of HSA as the molecule involved in *A. baumannii* response.

Keywords: Huma Serum Albumin, *Acinetobacter baumannii*, quorum sensing, iron, human fluids.

1. Introduction

Acinetobacter baumannii, a nosocomial and community acquired pathogen frequently resistant to multiple drugs, causes a wide variety of infections associated with high

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mortality rates. Highlighting the importance of *A. baumannii*, the CDC's 2019 Antibiotic Resistance Threats Report listed this pathogen into the urgent threat category [1]. This Gram-negative bacterium has also gained its reputation as a successful pathogen through its intrinsic antibiotic resistance, its metabolic adaptability, its ability to resist different stressors, and its high genomic plasticity [2-4]. A key feature of *A. baumannii*'s adaptability is its capacity to change its metabolism and nutritional needs [5,6]. Additionally, bacterial sensing of host environmental signals has been proposed to play a critical role in these adaptation processes. Studies examining the differential expression of specific genes of *A. baumannii* when exposed to different bodily fluids showed that *A. baumannii* could respond to these stimuli by shaping its pathogenic behavior [6-11]. Recent findings showed that when exposed to different human fluids, *A. baumannii* A118 displays large-scale complex responses affecting phenotypes highly relevant to bacterial persistence and infection [6-8]. For instance, human pleural fluid (HPF), a medium that primarily functions to lubricate pleurae during respiratory movements, triggers in *A. baumannii* a transcriptional response affecting a large number of genes related to metabolic processes [6]. Moreover, we observed that purified human serum albumin (HSA), which is the main blood protein component and an important component of HPF, indeed triggers a larger transcriptional response in *A. baumannii* A118, not only affecting natural transformation-related gene expression but also motility, efflux pumps, pathogenicity, and antibiotic resistance, among others [8,12,13]. We have also observed that strains with diverse degrees of pathogenicity respond differently to modifications of external conditions. For example, the mildly pathogenic strain A118 responds with larger transcriptional and phenotypic changes than other more pathogenic strains, when exposed to human fluids [6,7]. Supporting this observation, transcriptomic analysis performed for A118 and AB5075 strains exposed to cerebrospinal fluid (CSF) showed that AB5075 did not show significant changes at the transcriptomic level. In contrast, A118 showed differences in its transcriptome [13].

In the present work, we extend our previous observations and verify the distinctive and decreased transcriptional response of the hyper-virulent and extreme-drug resistant isolate AB5075 [14] exposed to HSA or HPF. This transcriptional analysis revealed a surprising response that was centered on genes associated with quorum sensing, fatty acid metabolism, motility, transport, uptake, and iron storage among others.

2. Results and Discussion

2.1. Hypervirulent and MDR A. baumannii transcriptome response to human fluids

The transcriptomic analysis of *A. baumannii* strain AB5075 revealed that HPF and HSA significantly affect the expression of 31 and 30 coding genes, respectively (FDR adjusted *P*-value of <0.05 and $\log_2$ fold change > 1). Eleven and 12 of these genes were up-regulated in the presence of HPF or HSA, respectively. The expression levels of 11 genes were modified in the presence of either fluid, with nine and two of them being up- and down-regulated, respectively. Genes whose expression was regulated by the presence of HPF or HSA encoded functions related to quorum sensing, fatty acid metabolism, motility, bacterial survival, efflux pump, biofilm, and functions associated with transport, uptake, and iron storage (Table S1). Nine out of the 11 genes responsive to the presence of HPF or HSA coded for functions related to quorum sensing (four) and fatty acid metabolism (five) (Table S1).

The number of *A. baumannii* AB5075 genes whose expression is modified by HPF or HSA was surprisingly low compared to HPF's effect on the mildly virulent strain *A. baumannii* A118, in which case more than 1,120 genes were differentially expressed experiencing a higher responsiveness to external stimuli [6,7]. The molecular and physiological bases behind these differences remain to be determined.

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2.2. Human fluids enhance the expression of genes involved in quorum sensing and quorum quenching in a hypervirulent and MDR A. baumannii strain

Analysis of mRNA extracted from *A. baumannii* AB5075 cells cultured in the presence or absence of HPF or HSA showed that the expression of four out of seven genes associated with quorum sensing and quorum quenching was significantly enhanced (Table S1 and Fig. 1A). The *kar*, *acdA*, and *fadD* genes code for 3-oxoacyl-ACP reductase, Acyl-CoA dehydrogenase, and Acyl CoA synthase, respectively. All three enzymes participate in AHLs synthesis [15]. The *aidA* gene codes for the quorum quenching α/β hydrolase AidA, which catalyzes the hydrolysis of the auto-inducer that results in inhibition of the motility and biofilm formation in *A. baumannii* [15].

To determine the role of HSA in promoting modification in levels of expression of quorum sensing related genes, we carried out quantitative RT-PCR (qRT-PCR) assays using total RNA extracted from *A. baumannii* AB5075 cells cultured in LB or LB supplemented with either HPF, HSA-depleted HPF (dHPF), or dHPF supplemented with HSA (dHPF + HSA) (Fig. 1B). All four genes, *aidA*, *kar*, *acdA*, and *fadD*, were up-regulated by up to 5-fold and 10-fold in medium supplemented with HPF or dHPF + HSA, respectively. No modifications in expression levels were observed when dHPF was added to the medium. These results strongly suggested that the HSA component of HPF is the molecule responsible for inducing the differential expression of these genes. Although *abaR* and *abaI* are not identified as differentially expressed by RNA-seq, qRT-PCR experiments showed significant increased expression in cells growing in medium supplemented with HSA (Fig. 1B).

To determine if the differential expression of the genes highlighted above is correlated with quorum sensing phenotypic modifications, we assessed the levels of acyl homoserine lactone (AHL) using the *Agrobacterium tumefaciens*-based solid plate assays [16]. The supernatants of *A. baumannii* cultures obtained in the presence of dHPF produced a blue color of high intensity, as it also observed with LB, indicative of the presence of large chain AHLs. Conversely, the supernatants from cultures containing HPF or dHPF + HSA produced low intensity or undetectable color, most probably caused by increased lactonase activity (quorum quenching) or reduced amounts of large chain AHLs (Fig. 1C). These results suggest that *a)* there is large chain AHLs-mediated communication between *A. baumannii* AB5075 cells and *b)* the large chain AHLs can be degraded, or its production inhibited by components present in HPF, such as HSA (Fig 1). The assays using culture medium supplemented with dHPF + HSA indicate that the component responsible for the low concentrations of large chain AHLs is HSA. Taken together the results generated by RNA-seq data, qRT-PCR analysis, and the quorum sensing phenotypic assays, we conclude that upon sensing the presence of HSA, *A. baumannii* AB5075 triggers a quorum quenching response. Determination of levels of short chain AHLs, carried out using the biosensor *Chromobacterium violaceum* based solid plate assays [17], showed no halos of violacein (Fig. S1). This was an expected result because it is known that the most predominant *A. baumannii* AHL is 3-hydroxy-C12-homoserine lactone [18-20].

It is noteworthy that while carrying out the *C. violaceum*-based solid plate assays, we observed a growth inhibition halo around the supernatant spot. This growth inhibitory effect was observed with supernatants from *A. baumannii* AB5075 cultures supplemented with HPF. Distinctive, yet smaller halos were also observed around locations where supernatants from cultures supplemented with dHPF or dHPF + HSA were spotted. No effect was observed when testing cultures in LB without any supplement (Fig. S1). This bacteriocin-like effect could give *A. baumannii* AB5075 a competitive advantage compared to other bacteria in the lung niche [21]. A direct analysis of the *A. baumannii's* secretome when growing in different conditions will be exceptionally informative. These experiments will help us to identify the mechanisms and molecule(s) responsible for this growth inhibitory effect.

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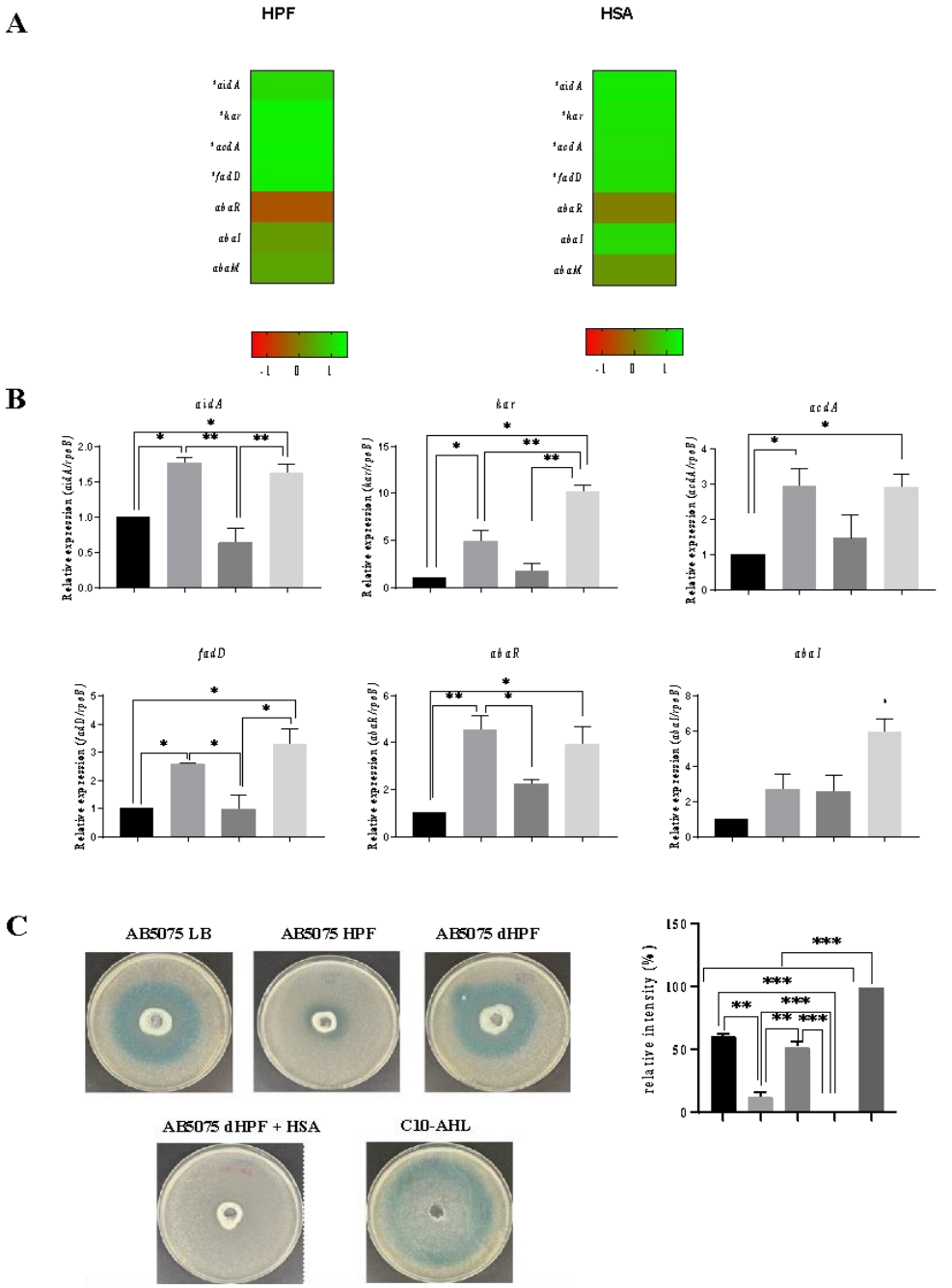

Figure 1. Phenotypic and genetic analysis of quorum sensing coding genes. (A) Heatmap outlining the differential expression of genes associated with quorum sensing in presence of HPF or HSA. The majority of quorum sensing associated genes are up-regulated (green) in the presence of HPF and HSA. The asterisks represent the DEGs (adjusted $P < 0.05$ with log2fold change > 1). (B) qRT-PCR of AB5075 strain genes associated with quorum sensing, *aidA, kar, acdA, fadD, abaR* and *abaI* expressed in LB or LB supplemented with HPF, dHPF, or dHPF + HSA. (C) Agar plate assay for the detection of AHL using *A. tumefaciens*. The presence of AHL were determined by the development of the blue color. Quantification of 5,5'-dibromo-4,4'-dichloro-indigo were estimated as the percentage relative to C10-AHL standard, measured with ImageJ (NIH). The mean ± SD is informed. Statistical significance ($P < 0.05$) was determined by ANOVA followed by Tukey's multiple-comparison test.

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2.3. Iron acquisition is modulated in presence of human fluids in the hypervirulent, MDR A. baumannii strain AB5075

As it is the case with numerous pathogens [22-25], previous research showed that the action of high-affinity iron-uptake systems contribute to *A. baumannii* pathobiology and virulence [26]. Upon entry into the human host, *A. baumannii* encounters extremely low concentrations of free iron as a consequence of the nutritional immunity [27-29]. The human host responds to the invasion of the pathogen reducing the already low free-iron levels in blood and tissue fluids triggering a set of reactions called the hypoferraemic response [22,30,31]. To survive, and thrive, under these conditions of iron starvation, like other bacterial pathogens, *A. baumannii* has developed several iron acquisition strategies, such as the production of different siderophores which are variably produced in different strains and likely account for Fe (III) scavenging from different sources [32].

Transcriptomic analysis of *A. baumannii* AB5075 showed that expression of nine genes coding for iron acquisition and metabolic functions was significantly reduced in the presence of HPF (Fig. 2A). The genes most inhibited by the presence of HPF were *feoA, bfnB, bfd, acbC, hemP, tonB, pfeA,* HMA *and exbD* and induced the expression of globin gene (Fig. 2A). The *feoA* gene belongs to the ferrous iron transport cluster (*feoABC*) [33] and proteins encoded by *bfnB and acbC* participate in biosynthesis of baumannoferrin, a hydroxamate siderophore [9,34]. The *tonB, exbD* and *pfeA* genes code for inner membrane protein complexes implicated in the transport of ferric-siderophores from the extracellular milieu to the cytosol [35]. The gene product of *hemP* is involved in the uptake of the iron source hemin [36] and *bfd* encodes a bacterioferritin-associated ferredoxin, a key component of the iron homeostasis machinery [37]. The HMA domain (heavy-metal-associated domain) is a conserved protein domain found in a number of heavy metal transport proteins [38]. The expression levels of *feoA* and *exbD* were confirmed to be statistically significant by qRT-PCR analysis (Fig. 2B). Both genes were reduced approximately 0.2- and 0.4-fold when the cells were incubated in medium supplemented with HPF or dHPF + HSA, respectively. A more significant reduction of the expression of *bauA*, which codes for the outer membrane receptor of ferric-acinetobactin complexes [39], and the gene coding for the heavy-metal-associated domain (HMA domain) protein was detected when the culture medium was supplemented with HSA (Fig. 2A).

The regulation of expression of *bauA* in different growth conditions was also determined by qRT-PCR. The results of these experiments show a statistically significant down-regulation in *bauA* expression in the presence of HPF or dHPF + HSA (Fig. 2B). The total Fe concentration in HPF is about 130 μM while that of LB, dHPF and dHPF + HSA is 89 μM, 99 μM, and 67 μM, respectively (as determined by a colorimetric iron assay kit, Sigma-Aldrich, USA) (Fig. S2 and Table S2). These results indicate that the inhibition of the expression of 14 of the 17 coding genes belonging to the *bas-bau* gene cluster (Fig. S3), a group of genes required for expression of the acinetobactin-mediated iron acquisition system [40], by the addition of HPF to LB could be due to the higher iron content of this host fluid because of the presence of significant concentrations of the iron chelating protein ferritin [41]. On the other hand, the increased expression of the *bas-bau* gene in the presence of HSA reflects the lower iron content of LB supplemented with dHPF or dHPF + HSA (Table S2). Furthermore, the expression of *tonB*, *pfeA* and *bfd* analyzed by qRT-PCR demonstrated statistically significant down-regulation in the presence of HPF, dHPF, or dHPF + HSA (Fig. 2B).

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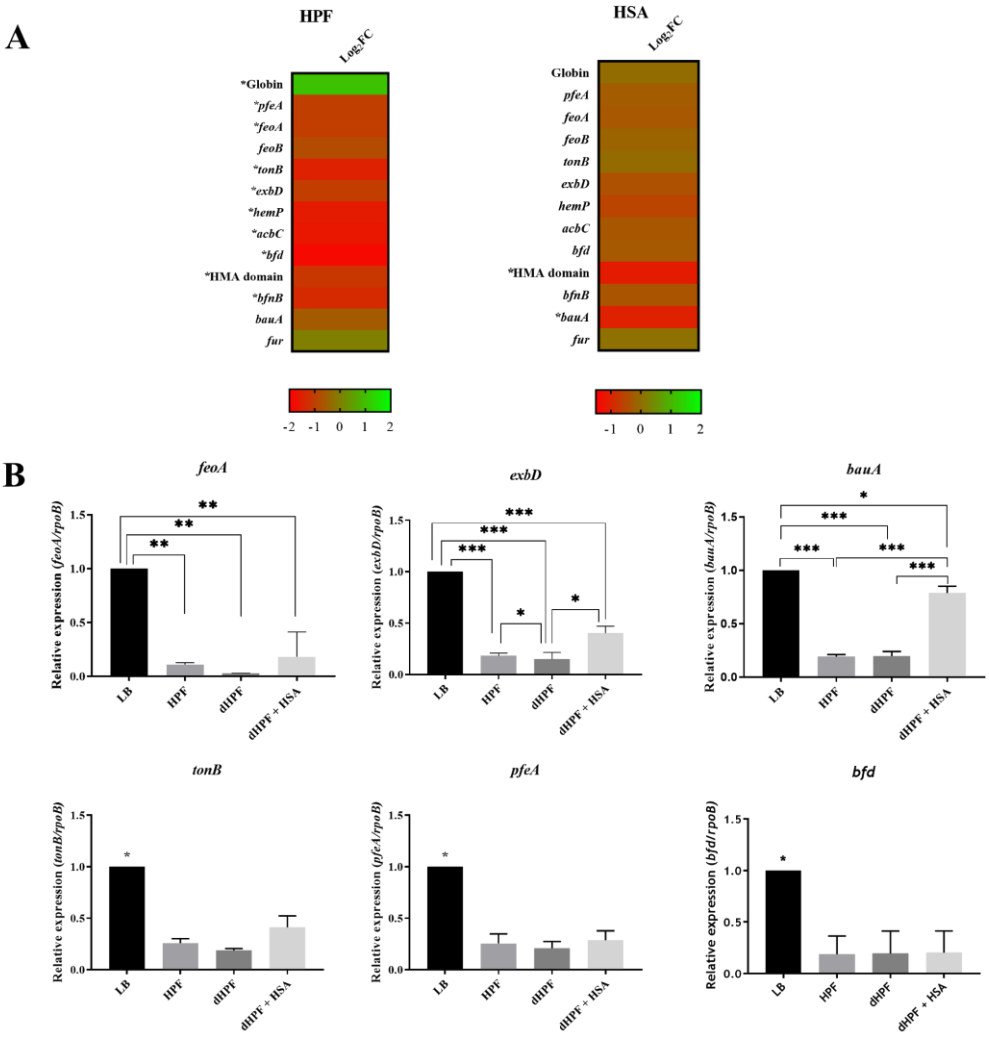

Figure 2. Phenotypic and genetic analysis of iron uptake genes. (A) Heatmap outlining the differential expression of genes associated with iron uptake in presence of HPF or HSA. The majority of iron uptake associated genes are down-regulated (red) in the presence of HPF and HSA. The asterisks represent the DEGs (adjusted *P*-value < 0.05 with log2fold change > 1). (B) qRT-PCR of AB5075 strain genes associated with iron uptake, *feoA, exbD, bauA, tonB, pfeA* and *bfd* expressed in LB or LB supplemented with HPF, dHPF, or dHPF + HSA. Fold changes were calculated using double ΔCt analysis. At least three independent samples were used, and four technical replicates were performed from each sample. Statistical significance ($P < 0.05$) was determined by ANOVA followed by Tukey's multiple-comparison test.

2.4. Human fluids shift fatty acid metabolic gene expression in A. baumannii AB5075

A. baumannii undergoes changes in its metabolism and nutritional needs under unfavorable conditions [5,6]. Genome-scale modeling of *A. baumannii* AB5075 in a murine infection model showed significant metabolic changes that help adaptation to the host during bacteremia. The metabolic changes observed were associated with the tricarboxylic acids cycle, gluconeogenesis, nucleotide and amino acid metabolism, as well as with biosynthesis of various cell components like peptidoglycan, lipopolysaccharide, and fatty acids [42]. Furthermore, there is recent evidence that the fatty acid β-oxidation pathway is associated with protection against host long-chain polyunsaturated fatty acids known to have antibacterial activity [43]. Three genes code for lipid biosynthetic functions. The *phaC*, which codes for a protein implicated in the synthesis of poly 3-hydroxyalkanoic acid [44], was significantly up-regulated in HPF and HSA conditions;

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the *pgaC*, which codes for a product implicated in synthesis of cell-associated poly-β-(1-6)-N-acetylglucosamine (PNAG) [45], was down-regulated in both condition, but statistically significant only in HSA condition; and the fatty acid (FA) hydroxylase gene, which is responsible of the hydroxylation of the carboxyl end of fatty acids [46,47], was statistically significant down-regulated in HPF and close to zero in HSA condition (Fig. S4A). The *scd* gene codes for an stearoyl-CoA 9-desaturase, enzyme used to produce the monounsaturated fatty acid oleic acid from the saturated fatty acid stearic acid. The presence of HPF or HSA showed that the expression of two *sdc* genes was significantly enhanced (Fig. S4A). The differential expression of *fadA, phaC, and scd* was further confirmed to be up-regulated by qRT-PCR analysis, which revealed up to 2-fold increase in expression in medium containing HPF and dHPF + HSA for *fadA and phaC,* and 2-fold increase in expression in medium containing dHPF + HSA for *scd* (Fig. S4B). Taken together, these support the role of HSA in modulating gene expression and a possible role in changes in fatty acid metabolism associated with the survival of *A. baumannii* within the host.

2.5. The catabolism of acetoin is altered in the presence of human fluids in A. baumannii AB5075

Acetoin (3-hydroxy-2-butanone) is an important molecule that prevents over-acidification of the cytoplasm as well as the surrounding environment resulting from the accumulation of acidic metabolic products [48,49]. The presence of HPF or HSA in the growth medium resulted in a not significant reduction in the expression of eight genes associated with the acetoin/butanediol catabolic pathway genes (Fig. S5A). However, qRT-PCR experiments revealed a statistically significant decrease in the expression of *acoA* in cells growing in medium supplemented with dHPF (0.113-fold, *P*-value: 0.0420). The same assays showed no difference in expression of *acoB* (Fig. S5B). Growth curves performed in M9 minimal medium containing 15 mM acetoin inoculated with overnight culture of AB5075 previously grew in LB, dHPF, HPF, or dHPF + HSA showed that of all four conditions *A. baumannii* AB5075's growth was most deficient in the presence of dHPF (Fig. S5C). Growth in minimal medium containing acetoin supplemented with overnight culture of AB5075 grown in HPF or dHPF + HSA was slightly slower when compared to LB media (Fig. S 5C). The data shown in this section are consistent with a role in growth modulation by acetoin when in combination with some human fluids.

2.3. Human fluids affect the expression of others important genes

We found genes associated to antibiotic resistance whose expression was significantly modified (Fig. S6A). An efflux pump-related gene was up-regulated in the presence of HPF or HSA. Conversely, the fluoroquinolone tolerance gene *dnpA* [50] and the colistin resistance gene *prmA* [51], were down-regulated by the presence of HSA (Fig. S6A). Genes associated to resistance to β-lactams were not modified by addition of HPF or HSA (Fig. S7). Other genes down-regulated in the presence of HPF were *wrbA, pspC and motA*. The *wrbA* and *pspC* genes code for stress regulators [52,53], and *motA* is a gene essential for motility [54] (Fig. S6A). Addition of HSA was associated to reduced expression of *katE* and *ywrO,* which code for a catalase and a general stress protein, respectively [55]. A gene belonging to the Hly-III family, which code for cytolysis associated functions (ref), was up-regulated by HSA (Fig. S6A). HSA also induced down-regulation of *csuB*, a component of the *csu* operon which is essential for *A. baumannii* biofilm formation [56] (Fig. S6A). Measurements carried out by qRT-PCR confirmed the reduced expression of *csuB* in medium containing HPF, dHPF, or dHPF + HSA. The variations in expression levels of *csuB* were 0.115-fold ($P < 0.0001$), 0.863-fold ($P = 0.0041$) and 0.164-fold ($P < 0.0001$), in HPF, dHPF or dHPF+HSA, respectively (Fig. S6B). The production of biofilm, a critical element for survival and persistence on abiotic surfaces

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and pathogenicity, was reduced when *A. baumannii* AB5075 cells were cultured in LB with addition of HPF, dHPF, or dHPF + HSA (Fig. S6C).

3. Materials and Methods

3.3. Bacterial strains

A. baumannii strain AB5075 was used as the model system. It is known to be to be highly virulent and resistant to antimicrobials [14,52].

3.2. RNA extraction, sequencing, and transcriptomic analysis

RNA extraction was done as previously described [8]. Briefly, AB5075 cells were cultured in Luria Bertani broth (LB) with or without 0.2 % HSA or 4% HPF and incubated with agitation for 18 h at 37°C. Overnight cultures were then diluted 1:10 in fresh LB broth and incubated with agitation for 7 h at 37°C. RNA was immediately extracted following the TRI REAGENT® Kit (Molecular Research Center, Inc., Cincinnati, Ohio, USA) as previously described [8].

RNA sequencing was outsourced to Novegene (Novogene Corporation, CA), where the RNA-seq library preparation (Illumina) and HiSeq 2500 paired-end 150 bp sequencing of three independent biological replicates in the presence or absence of HSA or HPF was performed. Trimming of low-quality bases at the ends of the reads to a minimum length of 100 bp and removal of Illumina adaptor sequences was performed using Trimmomatic [57] yielding an average of 7.6 million paired reads per sample. FastQC (www.bioinformatics.babraham.ac.uk/projects /fastqc/) was used to assess the quality of the reads before and after trimming. Burrows-Wheeler Alignment software (BWA) was used to align the RNA-seq reads to the genome of *Acinetobacter baumannii* AB5075 [58]. The alignments were visualized using the Integrated Genome Viewer software [59]. FeatureCounts [60] was used to calculate the read counts per gene, and differential expression analysis was performed using DEseq [61]. Features exhibiting FDR < 0.05 and $\log_2$fold change > 1 were considered statistically significant. Both RNA-seq data were deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE167117.

3.3. HSA depletion

To obtain HSA-depleted HPF (dHPF), 1 ml of HPF was placed into a 3 kDa Amicon™ Ultra Centrifugal Filter (Millipore, Temecula, CA, United States) and the solution was centrifuged at 20,000 x g for 10 minutes. To verify HSA depletion, an SDS-PAGE was conducted that contained 4% HPF and dHPF (Fig. S8).

3.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extracted and DNase-treated from *A. baumannii* strain AB5075 grown in LB and LB supplemented with 4% HPF, 4% dHPF, or 4% dHPF + 0.2% HSA, was used to synthesized cDNA using the manufacturer protocol provided within the iScript™ Reverse Transcription Supermix for qPCR (Bio-Rad, Hercules, CA, United States). The cDNA concentrations were adjusted to a concentration of 50 ng/µl. qPCR was conducted using the iQ™ SYBR®Green Supermix through the manufacturer's instructions. At least three biological replicates of cDNA were used and were run in quadruplet. All samples were then run on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States).

The transcript levels of each sample were normalized to the *rpoB* transcript levels for each cDNA sample. The relative quantification of gene expression was performed using the comparative threshold method $2^{-\Delta\Delta Ct}$. The ratios obtained after normalization were expressed as folds of change compared with cDNA samples isolated from bacteria cultures on LB. Asterisks indicate significant differences as determined by ANOVA

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followed by Tukey's multiple comparison test ($P < 0.05$), using GraphPad Prism (GraphPad software, San Diego, CA, United States).

3.5. N-acyl homoserine lactones (AHL) detection

Agrobacterium tumefaciens-based solid plate assays were carried out to detect AHL production [62]. Briefly, 500 µL of the homogenate were loaded in a central well of 0.7% LB agar plates supplemented with 40 µg of 5-bromo-3- indolyl-b-D-galactopyranoside (X-Gal) per ml and 250 µL (OD=2.5) of overnight cultures of *Agrobacterium tumefaciens* biosensor. The presence of AHL were determined by the development of the blue color [16,62]. As a positive control, 100 µl of N-Decanoyl-DL-homoserine lactone (C10-AHL) 12.5 mg/ml was utilized. Quantification of 5,5'-dibromo-4,4'-dichloro-indigo production in the different conditions, was determined by measuring the intensity of each complete plate, and substracting the intensity measured in the negative control, using ImageJ software (NIH). The values were normalized to the positive control, which received the arbitrary value of 100. Furthermore, *Chromobacterium violaceum*-based solid plate assays [17] were also carried out to detect short chain AHL. Briefly, 500 µL of culture supernatants of AB5075 cultured in LB or LB supplemented with 4% HPF, 4% dHPF, or 4% dHPF + 0.2% HSA, were loaded in wells made in LB plates overlaid with 5mL of a 1/100 dilution of an overnight culture of *C. violaceum* CV026 in soft agar (0.8%). Plates were incubated for 24 h at 30ºC and the production of violacein was examined.

3.6. Growth in the presence of acetoin

To test the ability of *A. baumannii* AB5075 to grow on acetoin as the sole carbon source, 1/50 dilutions of overnight cultures grown in LB, 4% HPF, 4% dHPF, and 4% dHPF + 0.2% HSA were inoculated in LB or LB plus 10 mM or 15 mM acetoin for 15 hours at 37°C with medium shaking. Growth was measured at an OD_{600} every 20 min using a Synergy 2 multi-mode plate reader (BioTek, Winooski, VT, USA) and Gen5 microplate reader software (BioTek).

3.7. Biofilm assays

Biofilms assays were performed as previously described [8]. AB5075 cells were cultured in LB or LB supplemented with 4% HPF, 4% dHPF, or 4% dHPF + 0.2% HSA with agitation for 18 h at 37°C. Experiments were performed in triplicate, with at least three technical replicates per biological replicate.

3.8. Determination of total iron concentration

Iron Assay Kit (Sigma-Aldrich) were used following the manufacturer's recommendations, to test the total iron concentration of the different media used in this work, LB or LB supplemented with 4% HPF, 4% dHPF, or 4% dHPF + 0.2% HSA.

3.9. Statistical Analysis

All experiments were performed at least in technical and biological triplicate. Data were expressed as means ± standard deviation. Statistical analysis using Mann-Whitney test or ANOVA followed by Tukey's multiple comparison test were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA), and a P value < 0.05 was considered statistically significant.

4. Conclusions

The data described here in this article support our previous observations in *A. baumannii* A118, a clinical isolate susceptible to most antibiotics and less virulent than the AB5075 strain [6-8,12,63]. This is an important observation that indicates that the role of HPF, with a strong role of HSA as the modulating molecule, may be considered general with respect to different *A. baumannii* strains.

A limited number of genes (31 and 30 HPF- and HSA-regulated genes, respectively), were modulated in the MDR and hypervirulent strain *A. baumannii* AB5075 compared to the susceptible strain A118 (1120 and 296 HPF- and HSA-regulated genes, respectively), reinforcing the concept of strain specific behavior already observed in previous works for *A. baumannii* [6-8]. In addition, this observation is in agreement with our previous work, when the phenotypic behavior and transcriptomic analysis of the AB5075 were studied in the presence of HPF or CSF, respectively (CC), showing least behavioral changes for the highly virulent strain.

The modulation of quorum sensing, quorum quenching, fatty acid metabolism, and iron acquisition system can contribute to the persistence of the bacterial exposure to human fluids allowing it to survive within the host. In sum, HPF components and distinctively HSA, can be sensed by *A. baumannii* strains with different degrees of pathogenicity modulating strain specific responses leading to persistence and survival when exposed to human fluids.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Table S1.** Genes associated with metabolic processes with differential expression in *A. baumannii* AB5075 under HPF or HSA induction; **Figure S1.** Agar plate assay for the detection of AHL in *A. baumannii* AB5075 exposed to HPF, dHPF, or dHPF + HSA using *C. violaceum*. **Figure S2 and Table S2.** Total Iron concentration in HPF, dHPF, and dHPF + HSA conditions studied. **Figure S3.** Heatmap outlining the differential expression of genes associated with acinetobactin production and utilization in presence of HPF or HSA. **Figure S4.** Differential expression of fatty acid metabolism genes. **Figure S5.** Differential expression of acetoin metabolism genes. **Figure S6.** Differential expression of significant genes in presence of HPF or HSA. **Figure S7.** Heatmap outlining the differential expression of genes related to beta-lactam resistance in presence of HPF or HSA. **Figure S8.** SDS PAGE showing the proteins present in LB, HPF, dHPF and dHPF + HSA conditions.

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