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Decrease of Staphylococcus aureus Virulence by Helcococcus kunzii in a Caenorhabditis elegans Model

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Social bacterial interactions are considered essential in numerous infectious diseases, particularly in wounds. Foot ulcers are a common complication in diabetic patients and these ulcers become frequently infected. This infection is usually polymicrobial promoting cell-to-cell communications. Staphylococcus aureus is the most prevalent pathogen isolated. Its association with Helcococcus kunzii, commensal Gram-positive cocci, is frequently described. The aim of this study was to assess the impact of co-infection on virulence of both H. kunzii and S. aureus strains in a Caenorhabditis elegans model. To study the host response, qRT-PCRs targeting host defense genes were performed. We observed that H. kunzii strains harbored a very low (LT50: 5.7 days ± 0.4) or an absence of virulence (LT50: 6.9 days ± 0.5). In contrast, S. aureus strains (LT50: 2.9 days ± 0.4) were significantly more virulent than all H. kunzii (P < 0.001). When H. kunzii and S. aureus strains were associated, H. kunzii significantly reduced the virulence of the S. aureus strain in nematodes (LT50 between 4.4 and 5.2 days; P < 0.001). To evaluate the impact of these strains on host response, transcriptomic analysis showed that the ingestion of S. aureus led to a strong induction of defense genes (lys-5, sodh-1, and cyp-37B1) while H. kunzii did not. No statistical difference of host response genes expression was observed when C. elegans were infected with either S. aureus alone or with S. aureus + H. kunzii. Moreover, two well-characterized virulence factors (hla and agr) present in S. aureus were down-regulated when S. aureus were co-infected with H. kunzii. This study showed that H. kunzii decreased the virulence of S. aureus without modifying directly the host defense response. Factor(s) produced by this bacterium modulating the staphylococci virulence must be investigated.

Keywords: attenuation, Caenorhabditis elegans, co-infection, Helcococcus kunzii, Staphylococcus aureus, virulence
INTRODUCTION

Diabetes mellitus is a worldwide public health problem representing the third cause of mortality and morbidity in the world (WHO, 2014). Foot ulcers are a common complication in diabetic patients. Indeed, 15–25% of diabetic patients will present foot ulcers during their life (Boulton et al., 2005). Infection of these ulcers is a frequent complication. It represents major causes of hospitalization, morbidity, and mortality. It is also one of the major causes of lower-limb amputation (Mayfield et al., 1998). Several studies have shown that diabetic foot ulcers (DFU) are polymicrobial (Dowd et al., 2008; Redel et al., 2013). However, *Staphylococcus aureus* represents the most frequent pathogen isolated in diabetic foot infections (DFI) (Gardner et al., 2013; Messad et al., 2013; Commons et al., 2015; Lesens et al., 2015; Dunyach-Remy et al., 2016; Hatipoglu et al., 2016). This Gram-positive coccus is a leading cause of a wide range of diseases from skin and soft tissue infections (e.g., impetigo, carbuncles) to life-threatening bacteremia, toxic shock syndrome, endocarditis, and osteomyelitis (Lowy, 1998), for which it deploys an arsenal of virulence factors to destroy host immune cells and tissues (Taconelli et al., 2006).

In DFI, *S. aureus* is associated with a great diverse community of bacterial species (e.g., enterobacteria, anaerobes, non fermentative Gram-negative bacilli, β-hemolytic streptococci, enterococci; Gardner et al., 2013). The transition between DFU and DFI is poorly understood. *S. aureus* can colonize and maintain the chronicity of the wounds but this state is transient. The knowledge of *S. aureus* pathogenicity reveals that this bacterium seems to be particularly adapted for soft tissue and bone infections. Indeed, the majority of infections remain localized to the feet notably in the toe bones (Dunyach-Remy et al., 2016). Social bacterial interactions are considered essential in numerous infectious diseases, including chronic wounds. These interactions have been described in all living entities (Brogeden et al., 2005). For example, a model of synergistic effect between uropathogenic *Escherichia coli* and *Enterococcus faecalis* showed that *E. faecalis* increased the virulence of *E. coli* (Lavigne et al., 2008). Moreover, translocation of several enterobacteria isolates in the bloodstream results in higher mortality (Pittet et al., 1993). Interactions involving clonal or divergent strains of the same species have also been described (Parsek and Greenberg, 2005; Tourret et al., 2011). However, this type of documentation of bacterial interaction is scarce in DFU/DFI. If metagenomic technologies have determined that distinct communities of bacteria are present at different sites of the body, challenges remain in understanding the complex interplay of these different species in contributing to modify the bacterial virulence (Price et al., 2009; Gardner et al., 2013; Fernandez et al., 2015).

Recently the emergence of new tools (e.g., mass spectrometry, DNA pyrosequencing) in bacterial identification has highlighted the frequent association between *S. aureus* and *Helcococcus kunzii*, a catalase-negative, facultative anaerobic Gram-positive coccus in DFU (Haas et al., 1997; Chagla et al., 1998; Riegel and Lepargneur, 2003; Dowd et al., 2008; Lemaitre et al., 2008; Park et al., 2014; Vergne et al., 2015). *H. kunzii* was first described as a non-pathogenic bacterium, likely member of the skin microbiome (Haas et al., 1997). This species is also known as an opportunistic pathogen that causes different types of infections (endocarditis, bacteremia, meningitis, breast abscess, wound infections, prosthetic joint infections, osteomyelitis) in immunosuppressed patients (diabetic patient, drug fiend, alcoholic; Chagla et al., 1998; Lemaitre et al., 2008; Park et al., 2014; Vergne et al., 2015). Nonetheless, the role of *H. kunzii* in the pathogenesis of cutaneous polymicrobial infections remains unknown. In this study, we sought to investigate the potential of virulence of *H. kunzii* strains isolated from DFU in a model of *S. aureus* induced infection of *Caenorhabditis elegans* (Irazoqui et al., 2010; Szabados et al., 2013; Visvikis et al., 2014; Messad et al., 2015). This model was previously used to study *S. aureus* virulence notably in strains isolated from DFU/DFI (Garsin et al., 2001; Sotto et al., 2012; Messad et al., 2015). Its pathogenicity in the worms was characterized by enterocyte effacement, intestinal epithelium destruction, and complete degradation of internal organs (Irazoqui et al., 2010) demonstrating the interest of this model in the study of bacterial-host interaction.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains studied are listed in Table 1.

A collection of 23 clinical isolates of *H. kunzii* collected from DFU in a multicentre study performed between February 2008 and August 2013 was used (Vergne et al., 2015). Moreover, to assess the co-infection between *H. kunzii* and *S. aureus*, in addition to the reference *S. aureus* strain Newman, two clinical *S. aureus* strains, both isolated and characterized in our hospital, were used: NSA1385 (a colonizing strain collected from uninfected ulcer) and NSA739 (an infecting strain collected from deep DFI; Sotto et al., 2008; Messad et al., 2015). *Escherichia coli* OP50 was used as control for nematodes. This bacterium is the standard feeding strain for Fer-15 nematodes. It harbors no known uropathogenic virulence factors.

The different bacteria were grown in Mueller-Hinton (MH) and Luria Bertani (LB) broth or agar at 37°C except *H. kunzii* strains which were grown on Columbia agar supplemented with 5% fresh sheep blood (bioMérieux, France) under a 5% CO₂ atmosphere at 37°C during 48 h.

*S. aureus*, *H. kunzii* and *E. coli* grew identically alone or in association on the nematode growth medium (NGM) used to worms experiments at 37°C (Figure S1).

Pulse Field Gel Electrophoresis (PFGE)

PFGE analysis of genomic DNA fragments of the 23 clinical isolates of *H. kunzii* was carried out after digestion with the restriction endonuclease *Smal*, as previously published for enterococci (Bourdon et al., 2011). The electrophoresis was performed using a CHEF-DR3III apparatus (BioRad, France) and PFGE patterns were interpreted according to well-established criteria (Tenover et al., 1995).
**TABLE 1** | Fifty percentage Lethal Time of *Caenorhabditis elegans* infected with different *S. aureus* and two representative *H. kunzii* strains and evaluation of feeding behavior by measuring the pathogen avoidance.

| Strain     | Characteristics of the strain (References) | LT50 in days (IC95% inf-sup) | Occupancy test after 16 h (%) | Density OP50 vs. others | Density NSA739 vs. others | Density NSA1385 vs. others | Density Newman vs. others |
|------------|---------------------------------------------|------------------------------|------------------------------|------------------------|------------------------|-------------------------|---------------------------|
| NSA1385    | *S. aureus*, clinical, colonizing (Sotto et al., 2008) | 4.7 (4.5–4.8)              | 98 ± 2                       | <0.001                 | <0.001                 | –                       | NS                        |
| NSA739     | *S. aureus*, clinical, infecting (Sotto et al., 2008) | 2.8 (2.4–3.0)              | 96 ± 4                       | <0.001                 | –                      | <0.001                  | <0.001                    |
| Newman     | *S. aureus*, reference                         | 4.3 (4.0–4.6)              | 95 ± 4                       | <0.001                 | <0.001                 | NS                     | –                        |
| H10        | *H. kunzii*, clinical, colonizing (Vergne et al., 2015) | 5.5 (4.6–6.4)              | 96 ± 4                       | <0.001                 | <0.001                 | <0.001                  | <0.001                    |
| H13        | *H. kunzii*, clinical, colonizing (Vergne et al., 2015) | 6.3 (5.8–6.8)              | 100 ± 0                      | NS                     | <0.001                 | <0.001                  | <0.001                    |
| OP50       | *E. coli*, control strain                     | 7.1 (6.6–7.7)              | 100 ± 0                      | –                      | <0.001                 | <0.001                  | <0.001                    |

The results are representative of at least five independent assays for each group of strains. *P* Pairwise comparison between LT50s using a log rank test; NS, not significant; LT50, 50% Lethal Time.

**Nematode Killing Assay**

The nematode infection assay was carried out as previously described using the Fer-15 mutant line (a temperature sensitive fertility defect; Lavigne et al., 2008). Overnight cultures of the studied bacterial strains in the NGM were harvested, centrifuged and suspended in phosphate buffered saline solution (PBS) at a concentration of $10^5$ CFU/mL. Ten microliters of these bacterial suspensions were inoculated on NGM agar plates. A ratio 1:1 concentration of $10^6$ was prepared during co-infection assays. To validate this ratio, we evaluated the CFU of both bacteria prior to the seeding onto agar to confirm that *S. aureus* and *H. kunzii* were present in an equal amount. The plates were incubated at 37°C for 8–10 h. Around 30 L4 stage nematodes per plate were thus seeded and then incubated at 25°C. An independent reader (blind to the culture) scored each day the number of live nematode under a stereomicroscope (Leica, France).

**Effect of Sequential Infection of *C. elegans***

*C. elegans* were infected with two representative *H. kunzii* strains (H10 with no virulence and H13 with a low virulence). After 12 h, 30 nematodes were transferred to NGM medium containing the different *S. aureus* strains. In the same way, to evaluate the bacterial persistence, nematodes were coinfected with *H. kunzii* and *S. aureus*. After 12 h, 30 nematodes were transferred to NGM medium containing OP50 strain. The conditions of nematodes preparation were strictly similar to previous assays described before. Final analysis established the Lethal time 50% (LT50), which corresponds to time (in days) required to kill 50% of the worms.

Three replicates repeated five times were performed for each studied strain.

**Feeding Behavior Experiments**

Firstly, all the studied bacterial strains were grown in LB broth media (with or without anaerobes conditions) at 25°C during 16 h. The cultures were then spotted on NGM plates. Around 30 L4 stage nematodes were deposited in the center of the bacteria lawn. To establish the occupancy assays, the number of nematodes inside or outside each lawn was counted after overnight incubation as previously published (Lavigne et al., 2013). The results were presented in percent occupancy (number of worms in the bacterial lawn on the total number of *C. elegans*). The experiments were performed in triplicate.

Secondly, we determined the number of bacteria within the nematode gut (Garsin et al., 2001; Lavigne et al., 2008). Briefly, nematodes were picked at 72 h, and the surface bacteria were removed by washing the nematodes twice in M9 medium containing 25 μg/ml gentamicin. The *C. elegans* were then mechanically disrupted in M9 medium containing 1% Triton X-100. Finally, after serial dilutions, 100 μl of the mixture were plated on LB agar medium and on Columbia agar supplemented with 5% fresh sheep blood (when the co-infection experiments were performed). The colonies [aspect and haemolytic activity (α haemolytic for *H. kunzii* and β haemolytic for *S. aureus*)] were counted after 24 h and the identification of each species was confirmed by MALDI-TOF (Vitek MS, BioMérieux). Three replicate assays were performed for each strain.

**Effect of *H. kunzii* on *S. aureus* Virulence Genes Expression**

Analysis of the mRNA levels of *spa*, *hla*, and *agr* was performed following the method previously published (Doumith et al., 2009; Kriegeskorte et al., 2014). These 3 genes are essential in the *S. aureus* pathogenicity notably in worms model (Sifri et al., 2003, 2006). *S. aureus* isolates and *H. kunzii* H13 were grown alone or in association in MH broth to an OD$_{600}$ of ~0.7. The total RNA extraction was performed using the RNeasy Mini kit (Qiagen, France) during exponential stages. Purity and concentration were determined by the NanoDrop 2000 spectrophotometer (Fisher Scientific, USA). The iScript<sup>™</sup> Select cDNA Synthesis Kit (Biorad, Hurcules, CA) was used to the synthesis of cDNA from 1 μg of total RNA for each sample. Real-time PCR were done in a LightCycler® 480 (Roche, France) using the LightCycler FastStart DNA Master PLUS SYBRGreen I kit, 100 ng of cDNA and 10 pmol of target primers (*Table 4*). Amplifications were analyzed in triplicate from three different RNA preparations. Cycle threshold (Ct) values of the different target genes were
compared with the Ct-values of the house-keeping gene (gyrB) (Sihto et al., 2014). The Newman strain was used as control. The normalized relative expressions of the studied genes were obtained for each strain following the equation: \( 2^{-\Delta \Delta Ct} = \frac{Ct_{\text{gene}} - Ct_{\text{gyrB}}^{\text{studied strain}}}{Ct_{\text{gene}} - Ct_{\text{gyrB}}^{\text{control strain}}} \) (Livak and Schmittgen, 2001). Results obtained for each gene were log-transformed to obtain a fold change difference between strains.

**Evaluation of Host Response by Quantitative Real Time-PCR**

For selected genes involved in *C. elegans* response against infection [hlh-30, lys-5, lgg-1, clec-7 (Visvikis et al., 2014), cyp-37BI (Irazoqui et al., 2010), sodh-1 (JebaMercy and Balamurugan, 2012)], transcript level analysis was performed by qRT-PCR following the same protocol described before. *C. elegans* were infected between 12 h with studied bacterial strains. The nematodes were then washed twice in water. Total RNA from animals was extracted by using TRIzol® RNA Isolation Reagents (ThermoFisher, France). The target primers used were presented in Table 4. The \( 2^{-\Delta \Delta Ct} \) method was used to analyze transcriptional changes in target genes using snb-1 as the housekeeping control gene (Livak and Schmittgen, 2001). Data analysis was performed with the Pfaffl method (Pfaffl, 2001). Error bars indicate the standard deviation (SD) of three independent experiments.

**Statistical Analysis**

Statistics and graphs were performed using GraphPad Prism 6.0 software.

For the nematode killing experiments, a log-rank (Mantel-cox) test was used to evaluate differences in survival rates between the different strains.

Log-transformed data were used for RT-PCR. The effects of bacterial infections on expression of selected genes involved in *S. aureus* virulence and in host response were performed using one-way ANOVA followed by Dunnett’s multiple comparisons test. A statistically significant difference was retained for \( P < 0.01 \).

**RESULTS**

**Virulence of *H. kunzii* and *S. aureus* Strains**

The virulence of a clinical panel of 23 *H. kunzii* isolates was evaluated in a nematode model. The genetic comparison of the 23 strains showed that the isolates were not clonally related (Figure S2) eliminating a clonal impact of the virulence behavior. Out of 23, 17 (74%) *H. kunzii* strains were non-virulent with a behavior similar to the non-pathogenic *E. coli* OP50 (LT50s: 6.1 days ± 0.4 vs. 7.1 days ± 0.5, respectively), a laboratory reference strain used to feed nematodes [\( P \)-value, non significant (NS)]. The other six *H. kunzii* strains (H7, H10, H17a, H20, H21, H22b) were significantly more virulent than OP50 (\( P < 0.001 \); Table 1, Table S1).

To compare the virulence between *H. kunzii* and *S. aureus*, we used well-characterized *S. aureus* strains: the *S. aureus* strain NSA739 (collected from DFI and harboring a high virulence potential), the *S. aureus* strain NSA1385 (collected from DFU and harboring a low virulence potential) and the *S. aureus* reference strain Newman (Sotto et al., 2008; Messad et al., 2015). We observed that the panel of *H. kunzii* presented significantly lower virulence than NSA739 (LT50: 2.8 days ± 0.4), NSA1385 (LT50: 4.7 ± 0.2) and Newman (LT50: 4.3 ± 0.3; \( P < 0.001 \)), respectively. The difference of virulence between the infecting and the colonizing strains of *S. aureus* was previously demonstrated (Table 1, Table S1; Messad et al., 2015). These results confirmed that *H. kunzii* strains are low- or non-virulent bacteria.

**Decrease of *S. aureus* Virulence by *H. kunzii**

When the different *H. kunzii* strains and NSA739 were used to co-infect *C. elegans*, an important attenuation of the *S. aureus* virulence was observed independently of *H. kunzii* virulence or non-virulence potential (Table 2 Table S2, Figure 1). The LT50 obtained with the strains coinfection varied between 4.1 and 5.7 days. They were significantly longer than the LT50 detected with *S. aureus* strain alone (LT50: 2.8 days; \( P < 0.001 \)).

To confirm the role of *H. kunzii* in the diminution of *S. aureus* virulence, we tested the effect of *H. kunzii* on two others *S. aureus* strains: NSA1385 (colonizing strain) and reference strain Newman and on the *E. coli* OP50. If the majority of *H. kunzii* strains had statistically no impact on *S. aureus* virulence, 8 strains (H4, H6, H8, H13, H16, H17b, H22b, H23) reduced significantly the virulence of NSA1385 (LT50: 5.6–6.3 days; \( P < 0.001 \); Table 3, Table S3). On the other hand, we observed that 6 *H. kunzii* strains (H9, H13, H18, H22a, H22b, H23) reduced the virulence of the reference strain Newman (LT50: 5.8–7.1 days; \( P < 0.001 \); Table 3). Interestingly no difference could be noted when worms were fed with *H. kunzii* + OP50 (Table 3), suggesting a specific effect between *S. aureus* and *H. kunzii*.

All these findings strongly suggest the important role of particular *H. kunzii* strains in the attenuation of *S. aureus* virulence isolated from wounds notably for highly virulent *S. aureus* strains.

**Effect on Feeding Behavior**

To exclude the possibility that the observed results in worms was due to a modification of their feeding behavior, an occupancy test was performed. None of the bacterial strains tested alone or in association presented strong avoidance behavior. No significant difference was noted in the fraction of nematodes on the bacterial lawn between the different associations studied (Tables 1–3, Tables S1–S3). We also measured the bacterial load in the intestine of nematodes at 72 h post infection (Lavigne et al., 2013). We found that all bacteria tested alone or in association can colonize and survive in the *C. elegans* intestine (Figure 2). The number of the *H. kunzii* and *S. aureus* CFU was around 4 × 10^5 bacteria per nematode (IC95% = 2.8–7.9 × 10^5) within the nematode intestine for each combination without statistical difference (\( P \)-value, NS).

These results confirm the low virulence of *H. kunzii* strains and suggest that the impact on the modulation of *S. aureus* virulence observed in *C. elegans* was not due to a modification of nematodes’ feeding behavior, nor to a reduction of *S. aureus*
The results are representative of at least four independent assays for each group of strains. P, Pairwise comparison between LT50s using a log rank test; NS, not significant; LT50, 50% Lethal Time. Infection of nematodes with H10 or H13 followed by transfer on OP50 or S. aureus 12 h after.

TABLE 2 | Fifty percentage Lethal Time of Caenorhabditis elegans co-infected with a virulent S. aureus strain (NSA739) and two representative H. kunzii strains and evaluation of feeding behavior by measuring the pathogen avoidance.

| Strain                  | LT50 in days (IC95% inf-sup) | Occupancy test after 16 h (%) | P OP50 vs. others | P NSA739 vs. others | P H10 vs. others | P H13 vs. others |
|-------------------------|------------------------------|-------------------------------|--------------------|---------------------|------------------|------------------|
| NSA739                  | 2.8 (2.4–3.0)                | 96 ± 4                        | <0.001             | –                   | <0.001           | <0.001           |
| H10                     | 5.5 (4.6–6.4)                | 96 ± 4                        | <0.001             | <0.001              | –                | NS               |
| H13                     | 6.2 (5.8–6.6)                | 100 ± 0                       | NS                 | <0.001              | <0.001           | <0.001           |
| H10+ NSA739             | 4.1 (4.0–4.3)                | 92 ± 5                        | <0.001             | <0.001              | <0.001           | <0.001           |
| H13+ NSA739             | 5.7 (5.3–5.9)                | 100 ± 0                       | NS                 | <0.001              | <0.001           | NS               |
| H10> + OP50             | 6.6 (6.2–6.8)                | 100 ± 0                       | NS                 | <0.001              | <0.001           | NS               |
| H13> + OP50             | 6.2 (5.8–6.6)                | 97 ± 3                        | NS                 | <0.001              | <0.001           | NS               |
| NSA739> + OP50          | 2.8 (2.4–3.0)                | 96 ± 4                        | <0.001             | NS                  | <0.001           | <0.001           |
| NSA1385> + OP50         | 4.4 (4.0–5.1)                | 100 ± 0                       | <0.001             | <0.001              | <0.001           | <0.001           |
| Newman> +OP50          | 4.0 (3.5–4.3)                | 94 ± 4                        | <0.001             | <0.001              | <0.001           | <0.001           |
| H10> + NSA739*          | 2.5 (2.4–2.7)                | 100 ± 0                       | <0.001             | NS                  | <0.001           | <0.001           |
| H13> + NSA739*          | 4.1 (3.7–4.4)                | 100 ± 0                       | <0.001             | <0.001              | <0.001           | <0.001           |
| OP50                    | 7.1 (6.8–7.7)                | 100 ± 0                       | –                  | <0.001              | <0.001           | <0.001           |

The results are representative of at least four independent assays for each group of strains. P, Pairwise comparison between LT50s using a log rank test; NS, not significant; LT50, 50% Lethal Time. Infection of nematodes with H10 or H13 followed by transfer on OP50 or S. aureus 12 h after.

infection rate or a hypothetical cytotoxicity effect of H. kunzii on C. elegans.

Transcriptional Host Response during Co-Infection between H. kunzii and S. aureus

To estimate the host response during co-infection between H. kunzii and S. aureus, we carried out qRT-PCR on six representative host defense genes of nematodes after infection by H. kunzii and S. aureus alone or in co-infection: hlh-30 (the key transcriptional factor-encoded gene), lys-5 and clec-7 (antimicrobial-encoded genes), cyp-37B1 and sodh-1 (cytoprotective and detoxification-encoded genes) and lgg-1 (autophagy-encoded gene). Of the different coinfection combinations we choose to study one H. kunzii isolate non-virulent in nematode model and reducing the virulence of all S. aureus strains (H13) and one with low virulence in nematode model and that had effect exclusively on NSA739 virulence (H10).

We found that nematodes fed with the two H. kunzii strains did not show significant differences of expression of host defense genes compared to nematodes fed with the non-pathogenic strain OP50. This result confirms that H. kunzii strains do not stimulate the C. elegans immune response (Figure 3). On the other hand, when nematodes were fed with the three S. aureus strains, they significantly overexpressed hlh-30, lys-5, sodh-1, and cyp-37B1 compared to nematodes fed with OP50 (P < 0.01). Only the autophagy gene lgg-1 had no modification of expression whatever the strain and the condition tested (P-value, NS). Interestingly, no significant difference in the expression of host response genes could be observed between each S. aureus strains tested (colonizing or infecting; Figure 3). These results confirm a nematode host response when C. elegans were infected with S. aureus.

Finally, when we co-fed nematodes with H. kunzii and S. aureus strains, we observed that the majority of host defense genes (hlh-30, lys-5, cyp-37B1, and sodh-1) were overexpressed compared to OP50 or H. kunzii alone (P < 0.01). Gene expression levels were equivalent to those observed with nematodes fed with S. aureus alone (P-value, NS; Figure 3). Surprisingly the clec-7 gene has only significant variation of expression when C. elegans were fed with the colonizing S. aureus strain NSA1385 (P < 0.05).

These results suggest that the co-infection with H. kunzii and S. aureus induced an overexpression of some host defense genes. However, the variation of expression of clec-7 gene during the coinfection of H. kunzii and the virulent/non-virulent S. aureus strains could suggest some modulations of...
host defense. So, during the coinfection, the attenuation of S. aureus virulence in presence of H. kunzii seems to not be due to its capacity to trigger C. elegans host response that would help the fight against S. aureus infection. H. kunzii seems to directly act in the modulation of S. aureus virulence and to have no major role on the modulation of host immune defense.

**Effect of H. kunzii on S. aureus Virulence Genes Expression**

To look into the possibility of direct attenuation of S. aureus virulence by H. kunzii, the expression levels of two representative virulence genes (*hla* and *spa*) and the main regulatory gene *agr* (that influences the expression of numerous S. aureus virulence genes) were measured for the different S. aureus strains associated...
with the H. kunzii isolate H13 and compared to those of S. aureus alone (Figure 4).

We observed that hla gene which encodes for the α-hemolysin (representing one of the most important virulence factors) was significantly derepressed in S. aureus NSA739 associated with H. kunzii H13 [Median −0.277; 95%CI (−0.41/−0.18); p < 0.01]. In the same way, agr was also significantly down regulated [Median −0.582; 95%CI (−0.36/−0.81); p < 0.001]. spa gene which encodes the protein A (representing one of the most important colonizing factor) showed a significant overexpression in S. aureus NSA739 associated with H. kunzii H13 [Median 0.197; 95%CI (0.15–0.27); p < 0.001]. The same results were noted for the two other S. aureus studied.

This data suggested that H. kunzii attenuated directly the virulence of S. aureus by a deregulation of virulence genes and the global regulator of this virulence.

Effect of Sequential Infections of C. elegans

To get a better understanding of the role of H. kunzii on S. aureus virulence, we evaluated the effect of sequential infections on nematodes. Firstly, we infected C. elegans with the different bacteria alone and in association during 12 h followed by a transfer of nematodes on OP50 strain. The results showed that S. aureus is clearly more virulent compared to H. kunzii (P < 0.001) and this virulence was not due to a constant reinfection since all the results were comparable to those obtained in the first experiments (Tables 2, 3).

Secondly, we sequentially inoculated the different associated bacteria. We infected C. elegans with the H. kunzii strains alone during 12 h followed by a transfer of nematodes on the different S. aureus strains tested. We observed that the LT50s for this protocol were significantly reduced (LT50: 2.5–4.1 vs. 4.1–5.7 days, respectively; P < 0.001). However, this impact was not clearly equivalent for the different combinations tested. Indeed, for the H. kunzii strain (H10) with different impact on the S. aureus virulence, the LT50 was similar to LT50 obtained for nematodes infected by NSA739 alone (LT50: 2.5 vs. 2.8 days, respectively; P-value, NS). For the H. kunzii strain (H13) with an impact on the virulence of all the S. aureus studied, LT50 remained significantly reduced compared to nematodes infected with NSA739 alone (LT50: 4.1 vs. 2.8 days, respectively; P < 0.001; Table 2). Thus, the direct association of H. kunzii and S. aureus has an impact on the attenuation of S. aureus virulence. This effect is significantly reduced or aborted when the infection is sequential suggesting the necessity to simultaneously co-infect with both H. kunzii and S. aureus to attenuate the virulence of S. aureus. H. kunzii seems to act directly on S. aureus reducing its virulence and thus the host response (showed by the reduction of cyp-37B1 and clec-7 expression previously).

**DISCUSSION**

Social interactions involving parasites, protozoa and prokaryotes have been frequently described (Tourret et al., 2011). Microbial co-occurrence networks indicate that bacterial species coinfect the same site of the human body and form microbial communities (Fernandez et al., 2015). However, documents concerning interactions between non-virulent and pathogenic microorganisms are scarce particularly in DFU. In this article we show for the first time evidence of the modulation of S. aureus virulence when associated with a commensal bacterium, H. kunzii, frequently found associated in chronic wounds of the lower limbs (Vergne et al., 2015).

Some studies described the interactions between S. aureus and other pathogens (Hoffman et al., 2006; Baldan et al., 2014; Nair et al., 2014; Zago et al., 2015; Frydenlund Michelsen et al., 2016). These interactions vary between the microorganisms: cooperation with E. faecalis and Candida albicans (Engelmann et al., 2011; Nair et al., 2014; Zago et al., 2015), competition with Lactobacillus sp. (Ortiz et al., 2014), and Streptococcus pneumoniae (Margolis et al., 2010). S. aureus can also have both interactions (competition and cooperation) with the same pathogen depending of the disease and the conditions such as Pseudomonas aeruginosa (Hoffman et al., 2006; Baldan et al., 2014; Serra et al., 2015; Frydenlund Michelsen et al., 2016). If P. aeruginosa seems to never coaggregate with S. aureus in chronic wound ulcers (Fazli et al., 2009), these bacteria could share some siderophores to favor the growth of each other (Harrison et al., 2008). Moreover, our team has recently demonstrated the coexistence of two S. aureus population on DFU notably one with a very low virulence potential (Messad et al., 2015). In this context, the study of the effect of H. kunzii is of particular interest. Although we confirmed that this microorganism has a low virulence potential in the nematode model (74% tested strains were non-virulent and 26% harbored a low-virulence profile), some studies have described that H. kunzii can also...
be an opportunistic pathogen (Lemaître et al., 2008), notably in chronic wounds (Riegel and Lepargneur, 2003; Moore et al., 2010; Stanger et al., 2015; Vergne et al., 2015). Its frequent association with S. aureus on DFU reinforced the need of a better understanding of the cooperation mechanisms between the two bacteria. Here, we observed that all the H. kunzii isolates associated with a virulent S. aureus strains (NSA739) clearly increased the lifespan of the C. elegans (LT50s: 4.1–5.7 vs. 2.8 days, \( P < 0.001 \)). This effect was confirmed when the nematodes were infected with H. kunzii and two other less virulent S. aureus...
strains (the reference strain Newman and a DFU colonizing strain NSA1385). Moreover, this effect seems to be specific to S. aureus while no effect could be observed when H. kunzii were associated with E. coli OP50. To explain these results, two hypotheses could be made: (i) H. kunzii modulated the immune response of C. elegans and help them to be more resistant or tolerant to S. aureus, (ii) H. kunzii modulated directly the S. aureus virulence.

Previous experiments showed that primary infection with S. aureus can increase vulnerability of C. elegans and modify its tolerance to an opportunistic pathogen Proteus mirabilis (JebaMercy and Balamurugan, 2012). The sequential infections of nematodes provide the evidence that H. kunzii does not affect the tolerance of C. elegans to S. aureus. The fact that nematodes tolerate more S. aureus when they are mixed with H. kunzii could be due to a direct interaction between the two strains. H. kunzii may have a direct effect on S. aureus by interfering with the expression of S. aureus virulence genes. To confirm this hypothesis, we analyzed the expression of main genes involved in nematode defense response after infection with S. aureus, H. kunzii, and both (Irazoqui et al., 2010; Visvikis et al., 2014). If H. kunzii strains did not modify these genes expression, S. aureus strains had a clear effect on the expression of C. elegans host defense genes particularly hli-30, cpp37, lys-5, and sodh-1, whatever the virulence of the strain. This observation is consistent with two studies showing that after 8 h of infection with S. aureus, C. elegans modified the production of defense genes (clec-71, sodh-11, cyp-37B1, lys-5) that have xenobiotic detoxification potential or antimicrobial activities, and then protect host by participating to host response (Irazoqui et al., 2010; Visvikis et al., 2014). Also, our results show that H. kunzii does not modulate the immune response of C. elegans and the effect observed was due to a direct interaction between H. kunzii and S. aureus virulence. The downregulation of hla and agr expression in S. aureus co-cultured with H. kunzii sustained this hypothesis. In presence of H. kunzii, S. aureus could be in a “colonizing” behavior (as suggested by the overexpression of spa gene). Taken together, our work also confirms that C. elegans are not just a simple model to study pathogens’ virulence. It is an entire organism that can establish immune mechanism to fight against infection and depending to the pathogen agent, can stimulate host defense genes (Irazoqui et al., 2010; Engelmann et al., 2011; Visvikis et al., 2014). Nematodes use some metabolic pathway of defense and express some genes that share similarities and/or homologies with those expressed during vertebrate and human infection (Irazoqui et al., 2010). Even if a low number of host and bacterial genes have been evaluated, the 6 selected C. elegans host genes and the 3 selected S. aureus virulence genes have been previously demonstrated as essential in the study of host-pathogen interaction (Sifri et al., 2003, 2006; Irazoqui et al., 2010; Visvikis et al., 2014). Further investigations need to be carried out in order to define by which mechanism(s) Helcococcus may alter S. aureus virulence.

To the best of our knowledge this is the first description of a virulence-modulating bacterial interaction between a non-virulent bacterial species and a naturally occurring pathogenic strain. This virulence attenuation was independent to host defense mechanisms in C. elegans model. We believe that this observation provides a new insight into S. aureus virulence. The possibility that a non-virulent commensal strain impacts the virulence of S. aureus is of great interest, considering the numbers of commensal bacteria contained in DFU (Gardner et al., 2013). Our results obtained in a model organism emphasize the importance of studying the connections between pathogenic species and the endogenous microbiota. If pathogenic bacteria are well-characterized in infection, they cannot be reduced to a single organism infecting host. All the bacteria participate to the chronicity of the wound at different levels and their virulence modulation has to be investigated to a best management of the wounds. The fact as a commensal bacterium decreases the virulence of clearly pathogenic bacteria could explain that S. aureus did not involve immediately an acute infection on chronic wound but rather remains in a biofilm status (which however induces a delayed healing) due to the different “environmental” conditions encountered by the pathogenic bacteria. Our results are a step in the understanding of the transition between DFU and DFI. This could also represent new ways to fight infections.

**AUTHOR CONTRIBUTIONS**

JPL, CDR, OV, EL, and AS conceived and designed the experiments. CNE, OV, MFG, VC, and CD performed the experiments. MFG, AV, VC, AL provided the Helcococcus strains. CNE, OV, MFG, EL, AS, JPL, and CDR analyzed the data. CNE, JPL, and CDR wrote the paper. OV, MFG, AV, VC, AL, EL, and AS reviewed and edited the manuscript.
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SUPPLEMENTARY MATERIAL
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