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Sickeningly Sweet: L-rhamnose stimulates *Flavobacterium columnare* biofilm formation and virulence

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Abstract

Flavobacterium columnare, the causative agent of columnaris disease, causes substantial mortality worldwide in numerous freshwater finfish species. Due to its global significance and impact on the aquaculture industry continual efforts to better understand basic mechanisms that contribute to disease are urgently needed. The current work sought to evaluate the effect of L-rhamnose on the growth characteristics of *F. columnare*. While we initially did not observe any key changes during the total growth of *F. columnare* isolates tested when treated with L-rhamnose, it soon became apparent that the difference lies in the ability of this carbohydrate to facilitate the formation of biofilms. The addition of different concentrations of L-rhamnose consistently promoted the development of biofilms among different *F. columnare* isolates; however, it does not appear to be sufficient as a sole carbon source for biofilm growth. Our data also suggest that iron acquisition machinery is required for biofilm development. Finally, the addition of different concentrations of L-rhamnose to *F. columnare* prior to a laboratory challenge increased mortality rates in channel catfish (*Ictalurus punctatus*) as compared to controls. These results provide further evidence that biofilm formation is an integral virulence factor in the initiation of disease in fish.

Keywords: biofilm, *Flavobacterium columnare*, iron uptake, virulence.

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Introduction

Columnaris disease, caused by the Gram-negative bacterial pathogen *Flavobacterium columnare*, continues to be one of the most detrimental bacterial diseases of freshwater fish throughout the globe. Columnaris disease is difficult to treat and severely impacts global aquaculture of catfish, salmonids, baitfish and other ornamental/aquaria-trade species (Sun *et al.* 2012; Declercq *et al.* 2013). The disease predominantly affects the external mucosal surfaces of fish and can lead to the rapid and widespread destruction of the gills, skin and fins. In the United States, channel catfish (*Ictalurus punctatus*) are an economically valuable farmed species which are highly susceptible to *F. columnare*, making them an important model

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for understanding the host–pathogen interactions that govern columnaris disease initiation and progression (Mohammed & Arias 2014). Intensive fish rearing systems are well suited for *F. columnare*, and in these settings, the pathogen is ubiquitous and opportunistic and outbreaks are particularly common when catfish experience stress due to factors including high rearing density, excessive handling or poor water quality (Chowdhury & Wakabayashi 1988; Wakabayashi 1991; Shoemaker *et al.* 2003; Farmer, Mitchell & Straus 2011).

F. columnare naturally occurs in a planktonic, free-living state where it can survive for long periods of time, even in the absence of nutrients (Welker *et al.* 2005; Mohammed & Arias 2014). In contrast, *F. columnare* also possesses the ability to form biofilms (Cai, De La Fuente & Arias 2013), the latter which are broadly defined as surface bound microbial communities inhabiting an organic matrix composed of autogenously derived extracellular polymeric substances (EPS; Jefferson 2004). The advantages of adopting this life stage are not completely clear for *F. columnare*, but biofilm formation could increase virulence in that it would; offer protection from desiccation, augment resistance to antimicrobials, improve nutrient acquisition, and protection against other bacteria (Branda *et al.* 2005; Sundell & Wiklund 2011; Rabin *et al.* 2015). In both *in vivo* and *ex vivo* experiments, adhesion patterns of *Flavobacterium* spp. onto gill tissue are characterized by the development of numerous mats or aggregates of bacterial cells (Decostere *et al.* 1999; Nematollahi *et al.* 2003; Olivares-Fuster *et al.* 2011; Declercq *et al.* 2015b).

Relatedly – on the host side of the equation – using RNA-seq approaches, previous work by our group demonstrated that differing carbohydrate binding lectin profiles in surface mucosa differentiate fish from families identified as resistant or susceptible to columnaris disease (Beck *et al.* 2012; Peatman *et al.* 2013). We have particularly focused on the role of a rhamnose-binding lectin (RBL1a) in facilitating *F. columnare* attachment and invasion in the gill. Transcriptomic profiling of the channel catfish gill revealed RBL1a expression was induced greater than 100-fold at 3 h following *F. columnare* experimental infection (Sun *et al.* 2012). The expression of RBL1a was shown to be positively correlated with host susceptibility to *F. columnare* infection. Follow-up studies revealed that by exposing fish to L-rhamnose or D-galactose immediately prior to an *F. columnare* challenge, both bacterial adhesion and catfish mortality were significantly reduced; presumably by the saturation of RBL1a binding sites by the presence of their native ligands (Beck *et al.* 2012). Taken together, these results suggested that host expressed RBLs were mediating *F. columnare* colonization of the channel catfish host. Given this newly identified importance of the RBL compartment, the influence of exogenous L-rhamnose exposure in modulating host susceptibility, and the linkage between carbohydrates and biofilm formation, we sought to gain insight into the effects of L-rhamnose exposure to *F. columnare* bacterial cells. Because prior studies have focused solely on the host response to L-rhamnose in an attempt to identify possible treatments and to better understand potential binding mechanisms, it was unclear whether this carbohydrate exerted any physiological effects on *F. columnare*. In this study, we reveal novel interactions between L-rhamnose and *F. columnare* in the context of biofilm development and virulence.

Materials and methods

Bacteriology

F. columnare isolates: LV-359-01 (low (LV) to moderately virulent (MV)) and LSU-066-04 (highly virulent (HV) in catfish (Beck *et al.* 2015b) and 94-081 (HV catfish isolate; Shoemaker *et al.* 2008; Soto *et al.* 2008) of the genomovar II classification, 0901393 (HV carp isolate) and CDI-A (low virulent (LV) carp isolate; Olivares-Fuster *et al.* 2007; Declercq *et al.* 2015a) of the genomovar I classification, the *Flavobacterium johnsoniae* isolate ATCC 29584 (Darwish, Farmer & Hawke 2008) and a *Pseudomonas fluorescens* catfish isolate (Stuttgart National Aquaculture Research Center; identified through 16S sequence analysis) were used for these studies. For all experiments, *F. columnare* isolates, LV-359-01 and LSU-066-04, *F. johnsoniae* ATCC 29584 and *P. fluorescens* (catfish isolate) were cultured in *F. columnare* growth medium (Farmer 2004; Darwish *et al.* 2008), while *F. columnare* isolates, 0901393 and CDI-A, were cultured in modified Shieh broth (Shieh 1980; Song, Fryer & Rohovec 1988). All isolates were retrieved from frozen glycerol stocks and streaked onto the appropriate

medium. After 24–48 h, bacterial colonies were dislodged from the agar using a sterile cotton swab and inoculated into FCGM or modified Shieh broth. Bacterial broth cultures were incubated for 24 h at 28 °C prior to use in all growth assays.

Bacterial growth of *F. columnare* with L-rhamnose

To determine total bacterial growth, overnight cultures of two isolates LV-359-01 and LSU-066-04 were diluted (1:100) in FCGM broth into separate tubes prior to the addition of 0, 10, 20, 40 or 80 mM of the carbohydrate L-rhamnose (Sigma-Aldrich) from a 1 M stock solution. From the individual tubes, eight 100 μL replicates were added to a 96-well polystyrene plate. Bacterial growth was evaluated by incubating the plate at 28 °C for 24 h and shaking briefly every hour prior to reading the absorbance at 550 nm using a Synergy H1 plate reader (Biotek).

Impact on biofilm formation

For biofilm assays, overnight bacterial cultures of *F. columnare* isolates LV-359-01, LSU-066-04, 94-081, 0901393 and CDIA, *F. johnsoniae* ATCC 29584 and *P. fluorescens* were diluted (1:100) as needed into fresh FCGM broth, modified Shieh broth, or sterile filtered well water into separate tubes prior to the addition of 0, 10, 20, 40 or 80 mM of L-rhamnose from a 1 M stock solution. From the individual tubes, eight 100-μL replicates were added to a 96-well plate and incubated statically for 24 h at 28 °C. To evaluate the impact of biofilms cultured under iron-limiting conditions, 100 μM of iron chelator (25 mM 2, 2′-bipyridyl, Sigma-Aldrich) was added 4 h after the inoculation of isolates LV-359-01 and LSU-066-04 with L-rhamnose into 96-well plates. In a separate experiment to evaluate an ability to overcome iron limitation, LV-359-01 plates were incubated with iron chelator and increasing doses of L-rhamnose for 24 h. One plate was assayed at 24 h for biofilm formation while the other was supplemented with 400 μM $FeSO_4$ and cultured for an additional 24 h at 28 °C and then assayed. Analysis of all microplate biofilms was carried out using a well-established staining protocol (O'Toole 2011). Briefly, after two washes and crystal violet staining of the 96-well plates, adherent bacteria stained with crystal violet were solubilized in 30% acetic acid and quantified by reading the absorbance spectrophotometrically at 550 nm using the Synergy H1 or Multiscan MS (Thermo Labsystems) plate readers.

In vivo effect of L-rhamnose on *F. columnare* virulence in catfish

Channel catfish reared at the Stuttgart National Aquaculture Research Center were maintained in 250-L tanks that received filtered well water and aeration from submerged air stones with a turnover rate of 2 h. In the first *F. columnare* laboratory infection, four replicates of 30 fish per treatment (300 g biomass $tank^{-1}$) were stocked into 18-L tanks containing 10 L of filtered well water. The water was provided through the ultra-low-flow water delivery system at a rate of 30 mL min^{-1} (Beck *et al.* 2015a,b). LV-359-01 was inoculated in 1 L of FCGM and cultured until an OD signal of 0.80 at 550 nm. Afterwards, the flask was removed and placed on a stir plate at room temperature. A 10 mL sample was removed for serial dilution and colony-forming unit (CFU) enumeration. Bath immersion challenges using LV-359-01 were conducted using calculated doses of $2-4 \times 10^8$ CFU mL^{-1} where L-rhamnose (40 or 80 mM) was added to the overnight planktonic suspensions 15 min prior to addition to the challenge aquaria. In laboratory, challenge experiment 1 (50 mL $aquaria^{-1}$) and challenge experiment 2 (75 mL $aquaria^{-1}$) of bacterial suspension (with L-rhamnose) were added per aquarium, and (no L-rhamnose) was added to the bacteria added to control tanks. An additional tank containing 30 fish was used as a no challenge control. The second bacterial challenge used the same number of fish and replicates per treatment, and no challenge control. Fish were not fed on the first day after bacterial challenges, but were subsequently offered pelleted catfish feed throughout the rest of the study (35% protein, 2.5% fat; Delta Western). Water quality parameters were evaluated as described (Beck *et al.* 2015b), and fish were observed twice daily at which time any dead or moribund fish were promptly removed and killed using Tricaine-S (Western Chemical). Animal care and experimental protocols were approved by the Harry K. Dupree Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and

conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

Statistics

Differences in total bacterial growth were assessed using a nonlinear regression model, and differences in biofilm growth were evaluated using Dunnett's multiple comparison. Survival data were analysed using Kaplan–Meier log rank survival curves. Probabilities of <0.05 were considered statistically significant. All statistics were performed using Prism 6.0 (GraphPad).

Results

Bacterial growth of *F. columnare* with L-rhamnose

Total bacterial growth was evaluated over a 24-h period after inoculating replicate cultures of isolates LV-359-01 and LSU-066-04 in FCGM broth supplemented with L-rhamnose. The absorbance values were plotted and a nonlinear regression model was used to determine whether there were differences between growth phases (Fig. 1). Using a best-fit curve and analysing the hill slope of the growth response curves, no differences were found in the overall exponential growth of LV-359-01 between L-rhamnose-treated samples or the control (Fig. 1a). Similarly, no differences were found in the exponential growth of LSU-066-04 in the L-rhamnose-treated samples or the control (Fig. 1b).

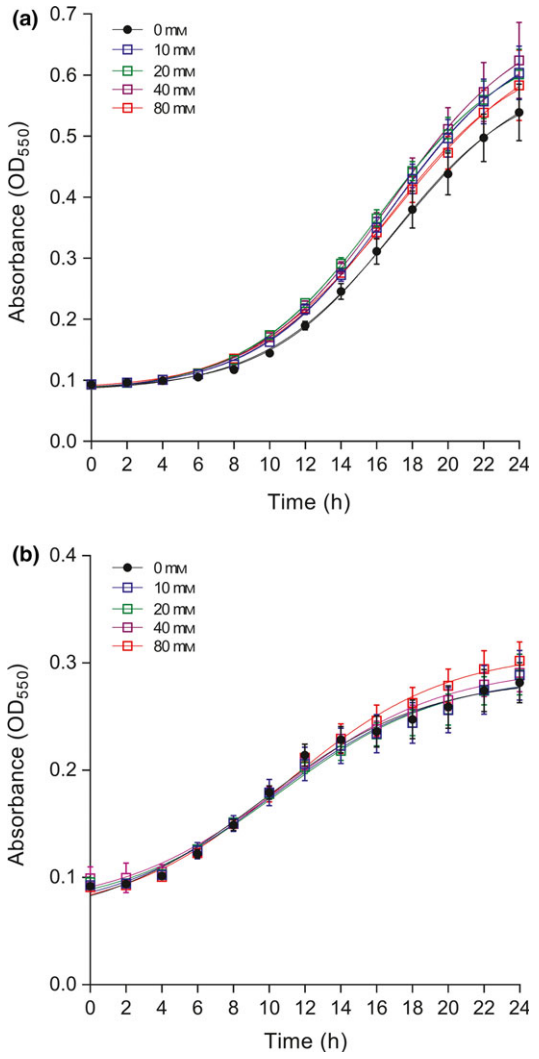

Figure 1 Effect of L-rhamnose on the total growth of *Flavobacterium columnare* isolates. Growth curves for LV-359-01 (a) and LSU-066-04 (b) cultured in *F. columnare* growth medium (FCGM) were shaken (200 rpm) at 28 °C and measured at OD_{550}. Data are represented as mean ± SDs ($n = 8$) for each time point. Control (closed black circles; 0 mM) and L-rhamnose (open squares; 10 mM blue, 20 mM green, 40 mM purple and 80 mM red). The coloured lines represent the 'best fit' dose–response growth curves of each concentration of L-rhamnose (best fit). [Colour figure can be viewed at wileyonlinelibrary.com]

Impact on biofilm formation

The previous analyses had sought to evaluate differences in total bacterial growth, which is a combination of both planktonic (free living) and biofilm-forming (adherent) bacterial cells. Recent work on biofilm formation in *F. columnare* demonstrated the ability of bacteria to adhere to many substrates (including tissues) and to develop biofilms under different physiological conditions (Cai *et al.* 2013). We therefore sought to determine if *F. columnare* adhesion and biofilm formation could be enhanced after the supplementation of L-rhamnose. A well-defined microplate assay was used to establish biofilms and then to determine whether there were any differences in biofilm development between treatments. Initial analyses of isolates LV-359-01 and LSU-066-04 showed that supplementation with L-rhamnose did in fact enhance biofilm formation (Fig. 2). Upon comparison of biofilms formed by isolates to which no L-rhamnose was added (0 mM, control); biofilm formation of LV-359-01 was significantly greater when treated with 80 mM

L-rhamnose ($P < 0.05$, Fig. 2a), and biofilms formed by LSU-066-04 resulted in a significantly higher OD signal when treated with 40 or 80 mM L-rhamnose ($P < 0.05$, Fig. 2b). Additional experiments were then conducted to further characterize this observation among other *F. columnare* isolates. Isolate 94-081 showed similar results to LV-359-01 in that it had formed significantly more biofilm when treated with 80 mM L-rhamnose ($P < 0.05$, Fig. 2c), and the results from isolate 0901393 were comparable to LSU-066-04 with significantly more biofilm formation when treated with 40 and 80 mM L-rhamnose ($P < 0.05$, Fig. 2d). In isolate CDI-A biofilm formation was significantly increased when treated with 20, 40 and 80 mM L-rhamnose compared to the control (0 mM L-rhamnose; Fig. 2e).

While no direct comparisons were made between the different *F. columnare* isolates due to different *in vitro* growth patterns, some similarities were observed. For instance, the OD values of the two carp isolates 0901393 and CDI-A in which only bacteria (0 mM, control) were cultured gave similar average OD values of 0.30 and 0.28, respectively. However, after the addition of L-rhamnose, the HV isolate (0901393) induced nearly twice as much biofilm signal (average OD value 0.55) after adding 80 mM of L-rhamnose compared to the average OD value (0.37) of the LV isolate (CDI-A) upon adding 80 mM of L-rhamnose. The same is true for catfish isolates: although similar average OD values (0.17) are seen in the controls, the average OD value of HV isolate LSU-066-04 (0.42) is higher than the

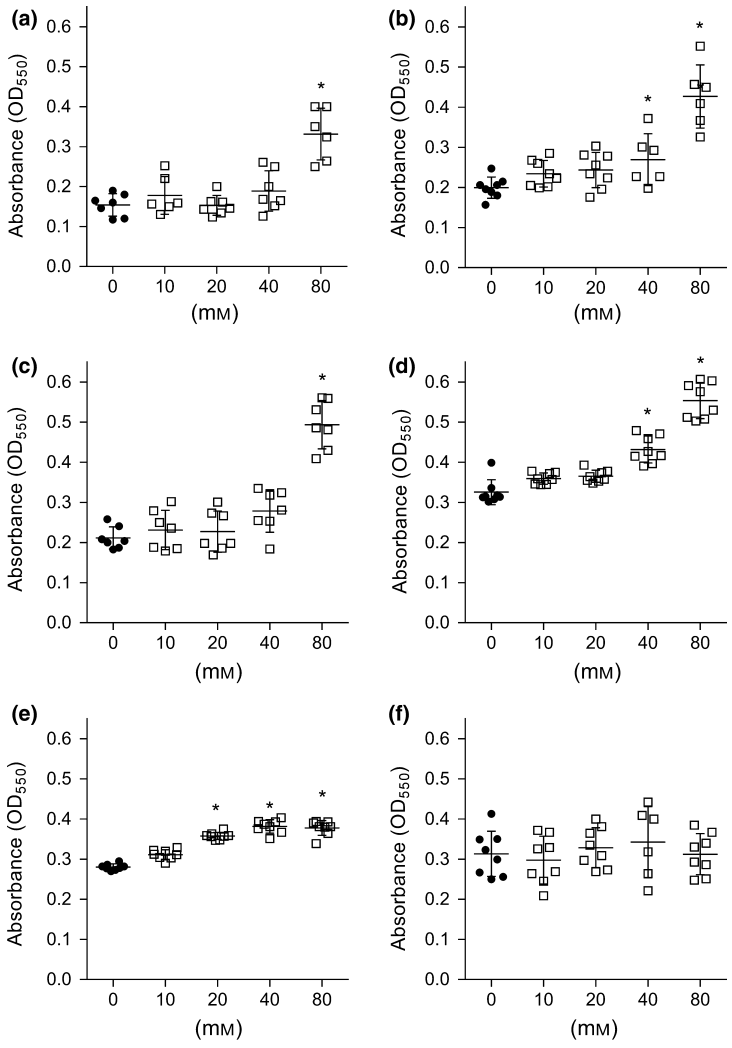

Figure 2 Effect of L-rhamnose on biofilm growth of *F. columnare* isolates. The biofilm growth of LV-359-01 (a), LSU-066-04 (b), 94-081 (c), 0901393 (d), CDI-A (e) and a *Pseudomonas fluorescens* isolate (f). Biofilms were cultured in FCGM or modified Shieh medium at 28 °C under static conditions for 24 h with different concentrations of L-rhamnose (mM) and measured at OD_{550} after crystal violet staining. Data are represented as mean ± SDs for the control (closed black circles, 0 mM) and L-rhamnose (open squares; 10 mM, 20 mM, 40 mM and 80 mM). Asterisk(s) denote a significant difference; $^*P < 0.05$ when compared to the control (0 mM).

average OD value of MV isolate LV-359-01 (0.33). For the HV isolate 94-081, the biofilm formation results are even more clear (average OD value of 0.2 in the control broth vs. average OD value of 0.5 in the broth to which 80 mM L-rhamnose was added).

From these results, it is clear that biofilm formation is stimulated after the addition of L-rhamnose to *F. columnare* planktonic cultures. Subsequently, to validate the biofilm assay and serve as a control for these experiments, a *P. fluorescens* isolate recovered from our facility was evaluated and it revealed that biofilm development does occur, but L-rhamnose does not enhance the formation of these biofilms in this bacterium (Fig. 2f). Results from another experiment using *F. johnsoniae* ATCC 29584 exhibited little to no biofilm formation even after supplementation with L-rhamnose (data not shown).

To further understand how L-rhamnose stimulates biofilm formation in *Flavobacterium* species, biofilm assays with both LV-359-01 and LSU-066-04 were performed; however, instead of culturing in FCGM, filter sterilized well water was used. Assays with water alone or L-rhamnose as the single carbon source resulted in no significant bacterial growth with either LV-359-01 or LSU-066-04 (data not shown), nor did we see any enhancement of biofilm formation when bacterial isolates were co-cultured with L-rhamnose (Fig. 3a–b). The next step was to evaluate how biofilm formation might be affected under other physiological conditions, for example supplementation with an iron chelator to perturb bacterial

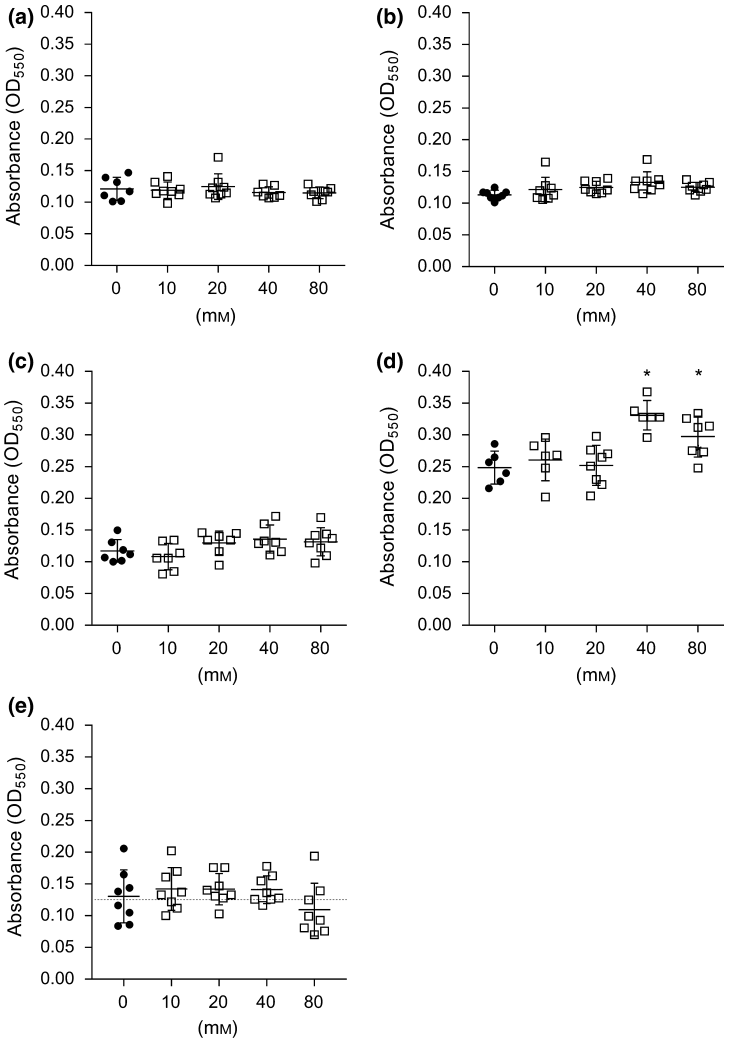

Figure 3 Effect of L-rhamnose on the formation of *F. columnare* biofilms under different physiological conditions. The biofilm growth of LV-359-01 (a) and LSU-066-04 (b) in water or after treatment with iron chelator, 100 μM 2′-2 dipyridyl, LV-359-01 (c) and LSU-066-04 (d). Panel (e), LV-359-01 biofilm was cultured for 24 h with iron chelator and L-rhamnose and was then supplemented with 400 μM FeSO4 for an additional 24 h. Dotted line in panel (e) represents the mean LV-359-01 biofilm growth after 24-h treatment with iron chelator and L-rhamnose. Biofilms were cultured at 28 °C under static conditions for 24 h (unless otherwise stated) with different concentrations of L-rhamnose (mM) and measured at OD_{550} after crystal violet staining. Data are represented as mean ± SDs for the control (closed black circles, 0 mM) and L-rhamnose (open squares; 10 mM, 20 mM, 40 mM, and 80 mM). Asterisk(s) denote a significant difference; $^*P < 0.05$ when compared to the control (0 mM).

growth of isolates LV-359-01 and LSU-066-04 (Beck *et al.* 2015b). Biofilm assays as described above (including L-rhamnose) were conducted for isolates LV-359-01 and LSU-066-04 and co-cultured with 2, 2′-bipyridyl. LV-359-01 planktonic growth occurred under these conditions (data not shown), but no biofilm was formed, not even when supplemented with L-rhamnose (Fig. 3c). Alternatively, LSU-066-04 showed that under the iron-limited conditions normal biofilm formation occurred and significantly more biofilms were formed following the addition of 40 and 80 mM L-rhamnose compared to the control (0 mM L-rhamnose; $P < 0.05$, Fig. 3d). As a final experiment, we tested whether LV-359-01 could overcome iron limitation by the addition of a 10-fold excess of $FeSO_4$ (Guan *et al.* 2013) after an initial 24-h period of 2, 2′-bipyridyl and L-rhamnose and assayed after an additional 24 h (Fig 3e). The LV-359-01 isolate was not able to overcome the iron-limited phenotype and remained unable to form biofilms.

In vivo effect of L-rhamnose on *F. columnare* virulence in catfish

Lastly, we sought to determine whether enhanced biofilm formation through stimulation with L-rhamnose could affect *F. columnare* infectivity or virulence. A challenge experiment with channel catfish through bath immersion was initiated. This first *F. columnare* challenge resulted in significantly greater mortality ($P < 0.05$) in the group challenged with isolate LV-359-01 (50 mL, no L-rhamnose) as compared to the no challenge control (Fig. 4a). LV-359-01 was stimulated with 40 mM L-rhamnose before being added to the challenge system also resulted in significantly greater mortality ($P < 0.05$) than the no challenge control. The addition of 40 mM L-rhamnose to the LV-359-01 stimulated overall higher mortality as compared to the fish challenged with isolate LV-359-01 (no L-rhamnose), but these two groups were not significantly different from one another.

The next challenge experiment which utilized a higher dose (75 mL) of LV-359-01 to further examine if there is an inherent difference between L-rhamnose-treated LV-359-01 and bacteria only groups (no L-rhamnose). This challenge supplemented LV-359-01 bacteria with 40 and 80 mM L-rhamnose prior to the infection, and revealed

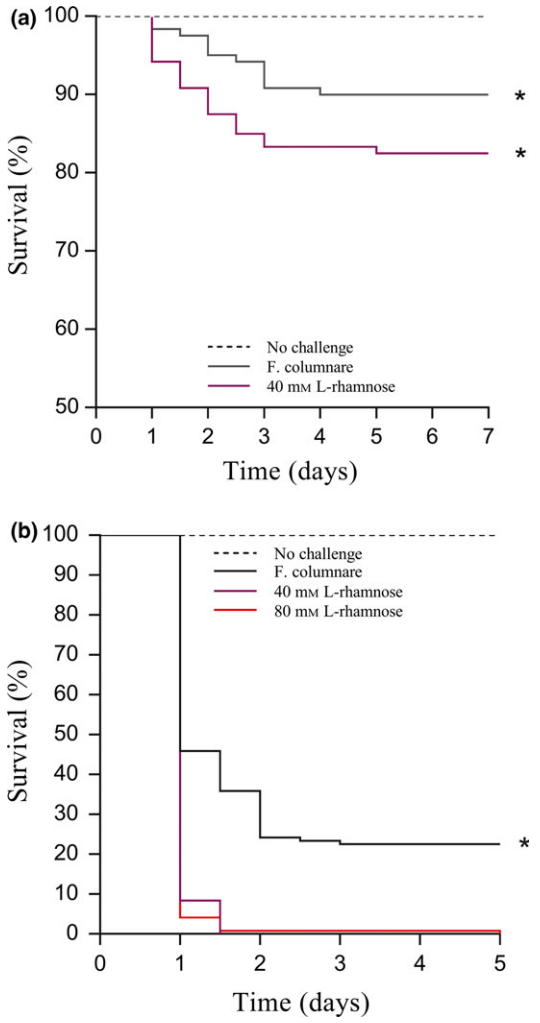

Figure 4 The impact of L-rhamnose on *F. columnare* virulence. Kaplan–Meier survival curves of channel catfish challenged with isolate LV-359-01 after the addition of L-rhamnose. Treatments are labelled in panels (a) and (b). Data represent cumulative mortality across four replicate tanks per treatment containing 120 fish total (30 per tank). Asterisk(s) denote a significant difference in survival as compared to the no challenge control (a) or *F. columnare* challenge (b); $*P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

that significantly greater mortality ($P < 0.05$) was observed in catfish challenged with rhamnose-treated LV-359-01 cultures (Fig. 4b). We observed nearly 100% mortality in both the 40 and 80 mM L-rhamnose-treated groups by day 1 while the LV-359-01 only group had only ~50% mortality at the same time. By day 5 the bacteria only group (no L-rhamnose exposure) had an overall mortality of 80%. Combining the results of the *in vitro* and *in vivo* trials demonstrate that

L-rhamnose can stimulate biofilm formation and contribute to *F. columnare* virulence.

Discussion

The current work sought to evaluate the effect of the carbohydrate L-rhamnose on the growth characteristics of *F. columnare*. Initially, no key changes were observed during the *in vitro* planktonic growth of the two *F. columnare* isolates in which bacterial growth was assayed when co-cultured with L-rhamnose. It soon became apparent that adding L-rhamnose to the culture broth impacts the ability to develop more robust biofilms in different *F. columnare* isolates from different fish species. Environmental *F. columnare* likely spends some of its life cycle in a planktonic state; however, to efficiently colonize, the gill or skin mucosae requires the attachment of this microorganism. The fish mucosae (primarily the gills and skin) are coated in a layer of mucus that serves as a primary barrier to an aquatic environment; wherein disruption of mucosal integrity often leads to bacterial infection (Li *et al.* 2013; Liu *et al.* 2013; Peatman *et al.* 2015). Historically, both *in vivo* and *ex vivo* experiments have demonstrated that adhesion of *Flavobacterium* spp. onto gill and skin tissues is characterized by the development of aggregates of bacterial cells or biofilms (Decostere *et al.* 1999; Nematollahi *et al.* 2003; Olivares-Fuster *et al.* 2011; Declercq *et al.* 2015b). Our results clearly suggest a pattern wherein L-rhamnose promotes the development of biofilms. In multiple *F. columnare* isolates, we demonstrate that *in vitro* biofilm development is enhanced after stimulation with L-rhamnose.

Free-living *F. columnare* cells can remain viable for long periods of time in inhospitable conditions and remain capable of recovery under different nutritive conditions (Arias *et al.* 2012); however, differing quality of aquatic environments and accessibility to nutrients can be very impactful in the formation of *Flavobacterium* species adhesion (Nematollahi *et al.* 2003; Cai *et al.* 2013). Because bacterial species often exploit multiple metabolic pathways to utilize different carbohydrate resources, we posited that perhaps the beneficial effect on biofilm formation could be due to an efficient use of this nutritive resource. Our results, however, showed that in well water L-rhamnose alone did not benefit total bacterial growth or biofilm development in *F. columnare* isolates LV-359-01 and LSU-066-04 when stimulated with L-rhamnose in the absence of any other nutritive sources. Suggesting that if an L-rhamnose catabolism pathway is present in *F. columnare* it does not by itself appear to be sufficient as a sole carbon source to promote biofilm formation.

These results began to support the concept that the transition from planktonic to biofilm growth goes beyond carbohydrate utilization; and it seemed more likely that the stimulation of biofilm development could be under genetic control. Some of the latest work in the field of biofilms has shown that biofilms likely form through the attachment of a combination of bacterial cells or cell aggregates, which are initiated through a number of distinct cellular changes (Kragh *et al.* 2016; Melaugh *et al.* 2016). It has previously been established that in *Pseudomonas aeruginosa* the transition to biofilm from planktonic cells generates multiple phenotypes and proteomic changes (Sauer *et al.* 2002). The control of the gene networks required for these phenotypes is carried out through the action of different families of transcription factors, where they often act to activate or repress the expression of many gene families (Ma, Buer & Zeng 2004; Janga, Salgado & Martinez-Antonio 2009). More specifically in *Escherichia coli*, roughly 115 transcription factors regulate cellular function including 32 that facilitate the expression of motility and biofilm formation operons (Martinez-Antonio, Janga & Thieffry 2008). In *E. coli*, L-rhamnose has been shown to be a strong transcriptional effector in the regulation of different rhamnose operons. The transcription factors (RhaS, RhaR) when complexed with L-rhamnose facilitate a cascade of rhamnose operons through their co-binding to upstream promoter regions and RNA polymerase (Egan & Schleif 1993; Wickstrum *et al.* 2010). L-rhamnose binding initiates conformational changes to the RhaS/RhaR proteins which facilitates their activity; less rhamnose binding results in less to no biological activity (Kolin *et al.* 2007). While there is variation in the amount of L-rhamnose necessary to initiate biofilm development, it is apparent there is a dose-dependent response to L-rhamnose in the formation of biofilms among the different *F. columnare* isolates that were tested. If L-rhamnose is activating a biofilm transcriptional pathway, a disparity between how much L-rhamnose is required to promote biofilm development could be due to differences in L-rhamnose uptake

efficiency among different isolates (Muiry *et al.* 1993; Rodionova *et al.* 2013).

There are multiple bacterial pathogens that cause disease as biofilms, including *P. aeruginosa*, *Klebsiella pneumoniae* and *Enterococcus faecalis* (Rabin *et al.* 2015); and the ability for biofilms to form is often directly correlated with the capacity to sense change in their environment and utilize different resources in the host milieu in order to initiate the disease process (Davey & O'Toole 2000; Jefferson 2004). There is a basic necessity for iron to facilitate a multitude of prokaryotic cellular processes. Two recent studies with *F. columnare* had shown the importance of iron acquisition machinery during planktonic cell growth and revealed the requirement for iron uptake as it relates to the virulence of the LV-359-01 and LSU-066-04 isolates (Guan *et al.* 2013; Beck *et al.* 2015b). Both of the latter studies demonstrated that the total growth of *F. columnare* in all isolates tested were impaired under iron-limited conditions. The work of Guan *et al.* (2013) also revealed the activation of iron uptake genes in *F. columnare* when under iron-limited conditions. In the study by Beck *et al.* (2015b), the LV-359-01 isolate was shown to be unable to cause disease when cultured in FCGM under iron-limited conditions, while LSU-066-04 under the same conditions proved to be as effective at causing disease as the LSU-066-04 control (no chelator). In the current work, we believe that iron acquisition is required for biofilm formation. This is reflected in the inability for LV-359-01 under iron limitation to develop biofilms in water or FCGM media in the presence of L-rhamnose, and for LSU-066-04 to retain the ability to scavenge for iron and sustain near normal growth and to have the benefit of better biofilm formation from L-rhamnose stimulation when grown in broth. Studies with *P. aeruginosa* have shown that EDTA kills bacterial cells and disrupts biofilm attachment, but also that free iron can protect these same biofilms from different chelating agents (Banin, Brady & Greenberg 2006). We then thought perhaps we could recover LV-359-01 from the iron-limited phenotype through the addition of $FeSO_4$; however, this also failed to promote biofilm formation when cultured in FCGM media. It seems likely that LV-359-01 may lack the ability to sufficiently regulate iron uptake under these conditions as opposed to LSU-066-04 which relies on siderophore- and ferroxidase-dependent systems to acquire iron from the environment. Upon activation of an iron-depleted LV-359-01 phenotype, a survival phenotype may prevent resources from going towards biofilm formation even when adequate iron is added into the system. Interestingly, the depletion of iron has been shown to inhibit *P. aeruginosa* biofilms through the disruption of quorum sensing and twitching motility systems (Cai *et al.* 2010), and more recent work has shown that *Capnocytophaga ochracea,* another Gram-negative bacterium involved in periodontal disease utilizes its gliding motility system to facilitate the formation of biofilms (Kita *et al.* 2016). While little to no work has been done on the gliding motility system of *F. columnare*, in the terrestrial *F. johnsoniae* a broad number of studies have described gliding motility and the T9SS mechanisms (McBride & Nakane 2015). There is no doubt that additional work will be required to dissect the different processes which facilitate *F. columnare* biofilm formation.

Virulence is simply defined as the ability to coordinate disease in an animal host. *F. columnare* virulence has traditionally been evaluated through the extent of mortality produced by different isolates through the use of various bath immersion disease models (Laanto *et al.* 2014; Beck *et al.* 2015b; Declercq *et al.* 2015a). Our data with LV-359-01 have shown that exposing bacteria to L-rhamnose prior to the challenge makes the bacteria more virulent. Our corollary for this is that L-rhamnose facilitates *in vitro* biofilm formation and in the *in vivo* challenge L-rhamnose-treated bacterial cultures contribute to more overall disease, possibly through the enablement for the bacteria to colonize the tissues and to form biofilms. However, when LV-359-01 growth is mired through iron limitation, there is no biofilm development, and under these conditions, there is little to no disease process (Beck *et al.* 2015b); therefore, biofilm formation is likely integral to virulence, and moreover, it is likely linked to the bacterium's ability to acquire iron. Our biofilm assay demonstrated similar stimulation patterns among carp *F. columnare* isolates 0901393 and CDI-A when stimulated with L-rhamnose; isolates that are designated as high and low virulence, respectively (Declercq *et al.* 2015a). However, the overall amount of biofilm produced when stimulated was quite different between these two isolates, possibly due to their different virulence profiles. After our

observation of the OD values of the *F. columnare* isolates, the more virulent the isolate, the higher the biofilm formation values upon addition of L-rhamnose across different host species. Recent work has also shown differences between the 0901393 and CDI-A isolates in their growth response and colony spreading morphology when exposed to the stress hormone cortisol, which may also be linked to the virulence of the different bacteria encountered (Declercq *et al.* 2016). The effect of different outside stimuli on *F. columnare* isolates could mean there are inherent differences in their ability to regulate biofilm formation, motility and could have wider implications on the capacity for quorum sensing (Rutherford & Bassler 2012). Future work using different *F. columnare* isolates will further benefit our understanding of how virulence is orchestrated to influence the disease process.

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