Probiotics to prevent *Staphylococcus aureus* disease?

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**ABSTRACT**

There are a plethora of probiotic formulae that supposedly benefit human health on the market. However, the scientific underpinnings of the claimed benefits have remained poorly established. Scientific evidence is now increasingly being provided that explains those benefits, for example, by immune-stimulatory effects or inter-bacterial competition between beneficial and pathogenic bacteria. In our recent study (Piewngam et al. *Nature* 2018), we show that *Bacillus* colonization of the human intestine is negatively correlated with that of the human pathogen, *Staphylococcus aureus*. This type of colonization resistance is achieved by secretion of a class of lipopeptides by *Bacillus* species that inhibits *S. aureus* quorum-sensing signaling, which we found is crucial for *S. aureus* intestinal colonization. Here, we discuss what these findings imply for the general role of *S. aureus* intestinal colonization, the role of quorum-sensing in that process, and potential alternative ways to control *S. aureus* infection.

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**INTRODUCTION**

It is now widely acknowledged that the ensemble of microorganisms that colonize human epithelial surfaces, the human microbiota, has a key role in determining human health. The number of microorganisms in the human gut exceeds by far that on any other body part, and intestinal colonization has, therefore, received the most attention. Some of the bacteria that colonize the human gastrointestinal tract are of considerable benefit to the host, while others may cause severe infections. A major reason for the expansion of pathogenic populations or establishment of foreign introduced pathogens, which ultimately can lead to infection, is the eradication of the healthy human microbiota by antibiotic consumption. Infection with *Clostridium difficile* (CDI), a leading cause of healthcare-associated morbidity and mortality worldwide, is a posterchild example of disease that is caused by the depletion of intestinal microbiota by antibiotics.

The contribution of the microbiota to host defense against pathogen colonization and overgrowth is called “colonization resistance” and it has been suggested to exploit mechanisms of colonization resistance by next-generation probiotics consisting of beneficial commensal bacteria. However, there are no FDA-approved colonization resistance-based therapies as of yet, which is mostly due to the fact that the mechanisms underlying bacterial interactions that lead to such resistance have remained poorly understood. Generally, the marketing and use of probiotics have far outpaced the scientific investigation of their alleged effects.

**Colonization resistance exerted by *Bacillus* against *Staphylococcus aureus***

*Staphylococcus aureus* is a dangerous human pathogen that can cause a variety of infections ranging from minor skin infection to life-threatening disease and fatal pneumonia and sepsis. Emergence and spread of methicillin-resistant *S. aureus* (MRSA) is particularly...
worrisme given the high rate and morbidity of MRSA infections in hospitals and the community.\(^8\) This situation is exacerbated by the lack of a working S. aureus vaccine, calling for the investigation of alternative treatment methods for this pathogen.

The predominant source for infection by S. aureus is previous asymptomatic colonization.\(^9,10\) Traditionally, the human nasal cavity has been considered the primary habitat for S. aureus.\(^11\) Approximately 20–30% of the healthy population are persistently colonized with S. aureus in the nose\(^12,13\) and S. aureus nasal colonization is a well-established risk factor for infection.\(^9\) However, S. aureus is also commonly found on other body sites such as the skin, throat, axillae, vagina, and especially the intestine.\(^11\) More recent studies have shown that intestinal colonization with S. aureus can persist following cessation of antibiotic treatment and forms an important reservoir for outbreaks of infectious S. aureus disease,\(^14,15\) which can perpetuate the spread of S. aureus in hospital wards and the community. Furthermore, S. aureus is a leading factor of food poisoning\(^16\) and has been discussed as a potential source of antibiotic-associated diarrhea.\(^11\) Nevertheless, as compared to the many studies that have addressed the molecular underpinnings of S. aureus nasal colonization, intestinal S. aureus colonization has remained severely understudied.

To investigate whether there are mechanisms of colonization resistance that can potentially be exploited to limit S. aureus intestinal colonization as a source for S. aureus infection, we collected fecal samples from 200 healthy individuals from rural Thai populations and performed 16S rRNA metagenomic sequence analysis to detect potential differences in the intestinal microbiota between S. aureus carriers and non-carriers.\(^17\) Nasal swabs were also taken. Of note, the observed rates of S. aureus nasal (13%) and intestinal (12.5%) colonization were considerably lower than those reported in the many previous studies that investigated primarily urbanized Western populations.\(^18\) To our initial disappointment, we detected no significant differences in the microbiome composition between S. aureus carriers and non-carriers using this 16S rRNA sequencing approach. However, using culture-based analysis we found a strong correlation between the presence of Bacillus spp. in the gut and the absence of both intestinal and nasal colonization by S. aureus: Fecal samples that grew Bacillus never grew S. aureus, and vice versa. That we could not detect this correlation using 16S rRNA sequencing-based analysis is not surprising given that such sequencing-based analyses are set up to detect high-order taxonomic shifts rather than specific differences on the species or genus level. This emphasizes the value of traditional, culture-based analyses to detect bacterial interactions even in the era of sequencing-based microbiome research.

In intensive follow-up analyses, we deciphered the mechanism underlying the observed colonization resistance (Figure 1). We showed that Bacillus species produce a family of lipopeptides, the fengycins, that have a strong capacity to inhibit the S. aureus quorum-sensing system Agr,\(^19\) which we revealed is indispensable for S. aureus colonization of the gut. Fengycins are cyclic lipo-decapeptides containing a frequently β-hydroxylated fatty acid with a sidechain length of 16–19 carbon atoms. They are known to specifically inhibit the growth of filamentous fungi while generally showing no antimicrobial activity against yeast and bacteria.\(^20\) The cyclic fengycin structure is reminiscent of the thiolactone ring structure of Agr extracellular signals (called AIPs for autoinducing peptides),\(^21,22\) and in fact, we showed that they work as competitors of AIP binding to their membrane receptor, AgrC, in all S. aureus Agr allelic groups (I–IV). Notably, feeding fengycin-producing (but not fengycin-deficient) Bacillus spores to mice completely eradicated S. aureus intestinal colonization. These results indicate a potential value of probiotic Bacillus to eradicate S. aureus colonization and of fengycins as quorum-sensing blockers for S. aureus infection. They also give new insight into the role of staphylococcal quorum-sensing for intestinal colonization and the relative role of different S. aureus colonization sites. We will discuss these implications in the following.

**Relationship between S. aureus nasal and intestinal colonization**

Despite several studies that have emphasized the widespread and considerable extent of S. aureus intestinal colonization,\(^18\) it has often been regarded as secondary to nasal colonization and possibly initiated by the nasopharyngeal-to-intestinal passage. It has also been speculated that the presence of S. aureus in the feces,
which is commonly used to determine the intestinal presence, is caused by colonization of the anorectal skin area rather than the intestine. However, adhesion of \textit{S. aureus} to intestinal mucus has been shown directly in human tissue\textsuperscript{23} and results from mouse studies by us and others are in support of the notion that there is genuine intestinal rather than only anorectal \textit{S. aureus} colonization.\textsuperscript{17,24}

Our results showing that an \textit{S. aureus} colonization resistance mechanism that is only present in the intestine also strongly impacts nasal colonization calls for a critical re-evaluation of the notion that the nose is pivotal to \textit{S. aureus} colonization. In fact, one possible explanation for our results is that nasal \textit{S. aureus} colonization is secondary and transient and the seemingly permanent nasal colonization is a consequence of frequent re-inoculation from a permanently \textit{S. aureus}-colonized intestine by anorectal-nasopharyngeal transmission. A more central than previously assumed role of the intestine for general human colonization by \textit{S. aureus} is not without further support. In addition to a series of reports suggesting that intestinal carriage is an important risk factor for \textit{S. aureus} infections,\textsuperscript{14,15,18} it has been shown that mice challenged intravenously with \textit{S. aureus} develop intestinal staphylococcal colonization, and fecal shedding results in \textit{S. aureus} transmission to cohoused naïve mice. Interestingly, patients who were both \textit{S. aureus} nasal and intestinal carriers were significantly more likely to develop \textit{S. aureus} infection than were those with nasal carriage only.\textsuperscript{15} Furthermore, intestinal \textit{S. aureus} colonization has been suggested to explain the failure of topical decolonization measures aimed solely at the nose.\textsuperscript{13,18} Moreover, staphylococcal skin and soft tissue infections are linked to rectal, but not nasal, colonization by MRSA strains in children.\textsuperscript{25} Finally, the sheer size of the intestinal as compared to nasal colonization site indicates that there are many more \textit{S. aureus} bacteria living in the intestine than the nose in a given individual, suggesting a much more extended reservoir for further spread. Altogether, these findings suggest that the intestine rather than the nose may be the dominating habitat of \textit{S. aureus} in humans from which re-colonization of other sites initiates. Ultimately, this hypothesis needs to be verified directly in humans.

\begin{figure}[h]
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\caption{Inhibition of Agr quorum-sensing in \textit{S. aureus} by \textit{Bacillus}. \textit{Bacillus} ssp. produce fengycin lipopeptides, which resemble the Agr auto-inducing peptide (made from the AgrD precursor by AgrB) and compete with the AIP for binding to the AgrC receptor, part of the AgrC-AgrA two-component system. This blocks signal transduction, which is dependent on AgrA phosphorylation and binding of AgrA to specific promoters, which include that driving agr\textit{ABCD} expression in an auto-regulatory fashion and toxin gene promoters in a direct or indirect manner. As a consequence, there is a diminished capacity to produce toxins and colonize the intestinal tract.}
\end{figure}
than those causing infection. This is in part due to the lack of animal colonization models that closely reflect the human situation. The mouse may be suited for the investigation of intestinal colonization at least to some extent, but the difference between mouse and human skin and also the nasal cavities are quite pronounced. To study nasal colonization, cotton rats have been used with somewhat more success. Overall, however, only a very limited list of molecular factors that impact *S. aureus* colonization of the nose and intestine have been identified, and the skin – while frequently investigated as a site of *S. aureus* infection – has remained virtually unexplored in that regard. These factors include teichoic acids and a series of cell surface-exposed binding proteins such as ClfB. The latter are believed to connect the bacteria to the nasal epithelium. Of note, mutants lacking *clfB* showed no defect in intestinal colonization, suggesting that the mechanisms underlying nasal and intestinal colonization by *S. aureus* differ significantly.

The Agr quorum-sensing system, while clearly regulating infectivity, had not been implicated in asymptomatic colonization before our study. In competitive experiments with equal amounts of *S. aureus* wild-type and isogenic *agr* mutant strain, we observed a dramatic impact of *agr* on intestinal colonization, with only wild-type *S. aureus* detected in the feces and the intestine. Furthermore, in a non-competitive experimental set-up, only those bacteria expressing the intracellular Agr effector RNAIII achieved colonization; *agr*-negative control strains never did. This extremely strong impact of Agr on intestinal colonization was quite unexpected. Commonly regarded as a negative regulator of surface binding proteins, Agr had not been among the likely candidates for colonization factors. However, we showed recently that in widespread clinical strains such as USA300, the notion that Agr reduces expression of surface-binding proteins does not universally hold true, and in particular the fibrinogen protein ClfA, which has been implicated in intestinal colonization, is in fact positively regulated by Agr in that strain. Then again, it is quite unlikely that fibrinogen binding plays a role for colonization of at least the healthy intestine and ClfA regulation by itself can explain the dramatic impact of Agr on intestinal colonization that we observed. Furthermore, we found that effect in three unrelated strains, suggesting that it is a more general feature of *S. aureus*. Altogether, the Agr-controlled factors that drive the impact of Agr quorum-sensing on intestinal colonization yet remain unidentified.

### Potential application of *Bacillus* probiotics and fengycins to control *S. aureus* infection

There are at least two possible routes for the translational use of our findings. First, live *Bacillus* may be used as a probiotic regimen to eradicate *S. aureus* colonization as a source for infection. Second, the fengycin quorum-sensing inhibitors may be used in synthetic or purified form as a quorum-sensing blocking drug directly against *S. aureus* infections (Figure 2).

*Bacillus subtilis* spores are a frequent component of probiotic formulae and also available as a stand-alone probiotic. A probiotic approach with *B. subtilis* would offer several advantages over other methods of decolonization. First, it is not antibiotic-based as the other methods that have been used to decolonize *S. aureus*, and thus does not come with the disadvantage of the extensive antibiotic use that would be necessary to decolonize a large subset of individuals or patients. In particular, for the decolonization of the intestine, antibiotic use is severely problematic due to the risk of infection with *C. difficile* and other pathogens that benefit from a broad eradication of the intestinal microbiota. Second, the Agr system is generally absent from Gram-negatives and not present in beneficial members of the human gut microbiota, while its presence in some other pathogenic bacteria may suggest even broader applicability of the *Bacillus* probiotic-based approach or the quorum-sensing blocker approach discussed below. Thus, the probiotic approach offers pathogen-targeted eradication that is not possible with antibiotics. Third, live *Bacillus* constantly produces the active substance, providing for a maintained presence that cannot be achieved even with multiple applications of drugs. Still, *Bacillus* is not a permanent colonizer of the intestine and our mouse data suggest that the probiotic would have to be given daily. Heterologous
production in a permanent beneficial colonizer is an alternative approach to consider achieving more permanent production of the inhibitor without the need for repeated oral application; yet, the permanent establishment of a genetically modified organism in the gut is likely not well received by many people. Fourth, the specific use of *Bacillus* spores as a highly resistant form of a probiotic organism also has the advantage of improved stomach passage – a considerable problem with many other probiotic supplements. After passage through the stomach, the spores germinate to produce metabolically active cells in the intestine. Lastly, *Bacillus subtilis* is generally considered safe and also reported to have additional probiotic benefits, which however except for a specific mechanism of immune stimulation in the intestinal epithelium remain poorly established scientifically.

While our results indicate that intestinal *S. aureus* eradication would have as a consequence a general decolonization of the human body, in principle a probiotic approach of interference could also directly be applied to the nose, if two prerequisites are met: (i) a beneficial colonizer can be found for heterologous expression of fengycins (or other *S. aureus* quorum-sensing blockers), and (ii) Agr proves to be as crucial for colonization in that body site as it is in the intestine. We believe such an approach to be superior to antibiotic- and also bacteriocin-based approaches that have been proposed recently, as a much more specific eradication of the pathogen can be achieved. This is hardly possible with antibiotics or bacteriocins that commonly have a broad target spectrum and also kill beneficial colonizers. We need to add that our current data indicate that other, potentially beneficial staphylococci are also targeted by fengycins, which is not a likely problem for the *Bacillus*-mediated intestinal application, but would have to be considered in approaches that target body sites such as the skin or nose where *S. epidermidis* and other staphylococcal species are abundant and supposedly play an important role in the stability of the healthy microbiota.

The other possible route of translational use comprises the application of fengycins as quorum-sensing blockers in an anti-virulence-based approach to control *S. aureus* infection. This is based on the fact that many *S. aureus* toxins, including phenol-soluble

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**Figure 2.** Potential therapeutic applications of *Bacillus* quorum-sensing blocking activity. Left, live *Bacillus* (subtilis) probiotics given orally in spore form germinate in the intestine to produce metabolically active cells that inhibit *S. aureus* quorum-sensing and thereby *S. aureus* intestinal colonization. Given the key role of intestinal colonization that our study implied, this may also lead to general decolonization including of the nose. Right, pure fengycin preparations may be given to control infection (e.g., lung, blood, or skin infection) in an anti-virulence quorum-sensing blocking approach.
modulins (PSMs), Panton-Valentine leucocidin (PVL) and other leukocidins, as well as α-toxin, are Agr-controlled and significantly impact S. aureus infection. This approach is often described as being less prone to the development of resistance as compared to traditional antibiotics, because genuine anti-virulence drugs do not kill the bacteria. However, the respective studies on resistance development are commonly only performed in vitro, and whether this holds true in an in-vivo setting with the selective pressure from efficient host defenses is debatable. Furthermore, many studies on quorum-sensing blocking drugs have not adequately ruled out growth effects that overlie the anti-virulence effect. Moreover, blocking quorum-sensing may not be advantageous in all infection types. Quorum-sensing blockers may be counterproductive in particular in biofilm infections. As for blood infections, the role of Agr is not entirely clear. While Agr-negative strains can be isolated at an increased percentage from patients with persistent bacteremia, strong contributions of Agr and Agr-regulated toxins to mortality in experimental sepsis argue in favor of a significant role of Agr in that disease type. Generally, quorum-sensing blockers are commonly seen as a valid alternative to conventional antibiotics that is worth considering at least in acute types of infection and in combination therapy with antibiotics. For S. aureus, this would include lung infections, and likely sepsis, as the most common causes of mortality due to S. aureus, as well as severe skin infections.

While still only in pre-clinical development, several quorum-sensing blockers have been described for S. aureus. Most of those, such as savirin, target the intracellular response regulator AgrA, an essential component of Agr. Fengycins, in contrast, work as competitive inhibitors of the extracellular Agr signal, and thus are active in the extracellular space. There are pros and cons associated with either type. Extracellularly active substances do not need transport into the cell and can thus be less hydrophobic, a considerable pharmacokinetic advantage. On the other hand, competitive inhibitors usually need to be applied in higher amounts. In that regard, the purified main fengycin species β-hydroxy-fengycin B had considerable activity in our study. However, a future detailed structure-function analysis of fengycins as Agr inhibitors and medicinal chemistry may lead to further improvement of activity.

Concluding remarks
While rigorous scientific evidence underlying the health benefits provided by probiotics is generally rare, those have been explained by several mechanisms, including modulation of the immune system, enhancement of the intestinal epithelial barrier, competition of bacteria for nutrients, and bacteriocin-mediated interference. Our study added direct interaction via a signaling system to this list and provided evidence obtained in humans indicating that a healthy diet that includes unsterilized vegetables is associated with the absence of a major pathogen. These findings suggest several translational approaches to limit S. aureus infection, among which the most elegant and promising appears the administration of a probiotic to limit intestinal and possibly general colonization with S. aureus. Whether this works and also can be extended to limit infection in hospital settings will be the subject of future clinical trials. Finally, whether fengycin-based therapy can be used for other pathogens that have Agr-like systems, such as C. difficile, is the subject of current investigation.

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