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BIOTECHNOLOGY

Roseobacter Group Probiotics Exhibit Differential Killing of Fish Pathogenic *Tenacibaculum* Species

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ABSTRACT Fish-pathogenic bacteria of the *Tenacibaculum* genus are a serious emerging concern in modern aquaculture, causing tenacibaculosis in a broad selection of cultured finfish. Data describing their virulence mechanisms are scarce and few means, antibiotic treatment aside, are available to control their proliferation in aquaculture systems. We genome sequenced a collection of 19 putative *Tenacibaculum* isolates from outbreaks at two aquaculture facilities and tested their susceptibility to treatment with tropodithietic acid (TDA)-producing *Roseobacter* group probiotics. We found that local outbreaks of *Tenacibaculum* can involve heterogeneous assemblages of species and strains with the capacity to produce multiple different virulence factors related to host invasion and infection. The probiotic *Phaeobacter piscinae* S26 proved efficient in killing pathogenic *Tenacibaculum* species such as *T. maritimum*, *T. soleae*, and some *T. discolor* strains. However, the *T. mesophilum* and *T. gallaicum* species exhibit natural tolerance toward TDA and are hence not likely to be easily killed by TDA-producing probiotics. Tolerance toward TDA in *Tenacibaculum* is likely involving multiple inherent physiological features pertaining to electron and proton transport, iron sequestration, and potentially also drug efflux mechanisms, since genetic determinants encoding such features were significantly associated with TDA tolerance. Collectively, our results support the use of TDA producers to prevent tenacibaculosis; however, their efficacy is likely limited to some *Tenacibaculum* species.

IMPORTANCE A productive and sustainable aquaculture sector is needed to meet the UN sustainable development goals and supply the growing world population with high-protein food sources. A sustainable way to prevent disease outbreaks in the industry is the application of probiotic bacteria that can antagonize fish pathogens in the aquaculture systems. TDA-producing *Roseobacter* group probiotics have proven efficient in killing important vibrio pathogens and protecting fish larvae against infection, and yet their efficacy against different fish pathogenic species of the *Tenacibaculum* genus has not been explored. Therefore, we tested the efficacy of such potential probiotics against a collection of different *Tenacibaculum* isolates and found the probiotic to efficiently kill a subset of relevant strains and species, supporting their use as sustainable disease control measure in aquaculture.

KEYWORDS *Tenacibaculum*, flavobacteria, roseobacters, probiotics, aquaculture, tropodithietic acid, *Phaeobacter*

The continuous growth of the human population poses a series of challenges in terms of efficient and sustainable production of high-quality foods. Fish and seafood constitute high-quality protein, and since the 1960s the seafood consumption per capita has doubled, and it is expected to increase even further in the coming years (1). To sustain this development, the aquaculture industry has grown concurrently, producing ~115 million tons of seafood, exceeding the production from wild catches (1, 2).

Bacterial infections caused by fish pathogenic bacteria are some of the most critical challenges in the efficient production of fish, which are often reared at high densities.

Editor Laura Villanueva, Royal Netherlands Institute for Sea Research

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The authors declare no conflict of interest.

Received 14 December 2021
Accepted 18 January 2022
Accepted manuscript posted online 26 January 2022
Published 22 March 2022

Members of the genera *Vibrio*, *Aeromonas*, and *Yersinia* are common pathogens in aquaculture, but, recently, flavobacteria from the *Tenacibaculum* genus have been observed as emerging fish pathogens in several fish farms (1, 3, 4). Here, *Tenacibaculum* causes tenacibaculosis in multiple species of finfish, including, but not limited to, red and black sea bream, trout, yellowtail, seabass, turbot, salmon, sole, and seabream (5–11). This broad-host-range pathogen represents a serious threat to a wide selection of marine aquaculture facilities worldwide. Our understanding of virulence factors involved in pathogenesis of *Tenacibaculum* spp. is limited and has been focused on *T. maritimum* (previously *Flexibacter maritimus* [12]), for which multiple genetic factors involved in the infection of fish have been identified through whole-genome sequencing (13). Within this species, factors relating to iron acquisition, degradation and utilization of host tissue, immune system evasion, and toxin production and excretion seem to be important. Furthermore, early symptoms of tenacibaculosis include scale loss, skin ulcerations, and inflammation where the formation of biofilms on the skin and gills is believed to be a prerequisite for infection (14). This is substantiated by the fact that the *T. maritimum* genome comprises a series of genetic determinants relating to host surface colonization such as adhesins, exopolysaccharide synthesis, and lectin or carbohydrate-binding motifs (13).

T. maritimum is susceptible to multiple antibiotics (11, 15, 16) and their application is a possible treatment of tenacibaculosis. However, the use of antibiotics poses a series of challenges, including poor efficacy when administered *in situ* (17), but also in terms of sustainable rearing practices. Extensive use of antibiotics, especially in smaller aquaculture facilities, has led to the spread of antibiotic-resistant bacteria in the environment and the spread of resistance determinants between aquatic bacteria and human pathogens (18–21). Therefore, sustainable alternatives to antibiotics are needed to ensure continued efficacy in the treatment of bacterial infections in humans and animals. One sustainable alternative is the development of vaccines. This, however, represents multiple caveats, since fish at the larval stage do not have adaptive immune systems, and vaccines exhibit high specificity to selected target serotypes. Vaccination attempts targeting *Tenacibaculum* spp. have only had limited success (22, 23) and, to our knowledge, the control of tenacibaculosis in the fish farming industry is currently limited to the administration of antibiotics with the exception of turbot, for which a vaccine is commercially available (16, 24). Another promising alternative disease control strategy is the use of probiotic bacteria that benefit the host health, often by antagonizing fish pathogens. One such group of potential probiotic bacteria is the tropodithietic acid (TDA)-producing roseobacters. These can kill or reduce the growth of several pathogens from the *Vibrio* genus in multiple different aquaculture relevant systems (25–28). Furthermore, they can improve survival of fish larvae and live feed challenged with pathogenic vibrios (29, 30). The antimicrobial compound TDA acts as a proton antiporter (31) and likely interacts with multiple targets in the cell, ultimately rendering fast-growing pathogens such as vibrios iron- and energy-depleted upon exposure (32). The numerous targets of TDA may be a contributing factor to the observation that the development of resistance is not readily inducible in susceptible organisms (33, 34). However, a substantial number of marine bacteria coinhabiting niches alongside TDA-producing roseobacters have a natural tolerance to TDA (35) and may even exhibit increased abundances in the presence of TDA producers in some cases (36). It is currently unknown to what extent the control of tenacibaculosis with roseobacter probiotics is a viable alternative to treatment with antibiotics and, hence, the aim of the present study is to determine to what extent TDA-producing roseobacters are capable of antagonizing different species of the emerging fish pathogenic *Tenacibaculum* genus.

RESULTS

A total of 48 putative pathogenic *Tenacibaculum* isolates was obtained from disease outbreaks in two aquaculture facilities (F1 and F2) rearing Senegalese sole and seabass/seabream, respectively. An initial screening based on full-length 16S rRNA gene

sequences indicated that 19 were flavobacterial isolates (three from F1 and 16 from F2). Fifteen belonged to the *Tenacibaculum* genus, and four belonged to the closely related *Joostella* genus (see Table S1 in the supplemental material). The remaining isolates comprised members of the *Vibrio*, *Shewanella*, *Litoreibacter*, *Pseudoalteromonas*, *Bacillus*, and *Epibacterium* genera. Whole-genome sequencing of the 19 flavobacteria and subsequent extraction and BLAST-analyses of the 16S rRNA genes confirmed that from F1, all three flavobacterial isolates belonged to the *T. soleae* species (Fig. 1A). From F2, the isolates represented *T. soleae* (three isolates), *T. mesophilum* (four clonal isolates), *T. discolor* (four isolates), *T. gallaicum* (one isolate), and *Joostella atrarenae* (four clonal isolates). Strains within the *T. soleae* and *T. discolor* species exhibited substantial genomic heterogeneity (Fig. 1A), and while the closest known relative to one of the *T. discolor* isolates, F2_11, was a *T. discolor* type strain according to the TYGS database, it was below the species-level cutoff in both an average nucleotide identity analysis (ANI; Fig. 1A) and in the TYGS comparisons, suggesting that this could be an undescribed species closely related to *T. discolor*. Due to the lack of representative sequences in the TYGS database, the identity of the *T. gallaicum* and the *J. atrarenae* could not be established beyond 16S rRNA gene phylogeny. Overall, the outbreak at the F2 facility involved a more diverse collection of potential flavobacterial pathogens.

Virulence factors and biofilm formation. Multiple genes encoding putative virulence factors were observed in the genomes of members of the *Tenacibaculum* genus, corroborating the assumption that these flavobacteria were involved in the disease outbreaks, however, a homolog to the *stp* gene, encoding a serine-threonine phosphatase (STP) presumed to be important for survival in the infected host, was the only virulence factor identified in the *Joostella* isolates from F2 (Fig. 1B). In contrast, this gene was not observed in any of the *Tenacibaculum* isolates. The most commonly observed virulence factors among *Tenacibaculum* isolates related to iron acquisition factors, manganese-dependent superoxide dismutases (SodA), heavy metal resistance factors, and exopolysaccharides and adhesins involved in biofilm formation (Fig. 1B).

Isolates of the *T. soleae* species (F1_1, F1_2, F1_3, F2_7, F2_22, and F2_25) represented three different clades (Fig. 1), all carrying virulence genes involved in iron acquisition, exopolysaccharide production, superoxide dismutase production, and heavy metal resistance. In addition, genes encoding collagenases for the degradation of host tissues were unique to the *T. soleae* species, and yet it was only observed in two of the three clades (Fig. 1B). In contrast to the other members of the species, one clade represented by two strains, F2_7 and F2_22, lacked genetic determinants involved in capsule formation, while they, on the other hand, carried genes relating to adhesin production, a trait not observed in the other clades within the species (Fig. 1B). Similarly, the *T. discolor* isolates (F2_11, F2_13, F2_16, and F2_20) represented three different clades. Members of all three clades harbored genes relating to iron acquisition and superoxide dismutase production, and in contrast to other members of the genus, all *T. discolor* genomes encoded a secretion system homologous to the Por system (type IX secretion system; T9SS) from the fish pathogen *Flavobacterium psychrophilum*. The strain F2_20 lacked the adhesin homologs present in the other *T. discolor* isolates and carried heavy metal resistance determinants (Fig. 1B). *T. mesophilum* isolates were highly genetically homogeneous, and all carried virulence factors relating to iron acquisition, exopolysaccharide and adhesin production, superoxide dismutase production, heavy metal resistance, and arsenate reductase production.

Biofilm formation is believed to be a key virulence factor in the early stages of infection by *Tenacibaculum* spp. Furthermore, biofilm formation in *T. maritimum* may facilitate persistence and reinfection in aquaculture facilities, and hence we sought to determine whether the isolates were proficient biofilm formers *in vitro* compared to the probiotic strain using a modified O'Toole-Kolter biofilm assay. Biofilm formation was observed for several isolates, although the ability to form biofilm was highly species and/or strain-specific (Fig. 2). The type strain *T. maritimum* DSM 17995 was the most proficient biofilm former of the tested flavobacteria, but none of the isolated pathogenic species, nor the

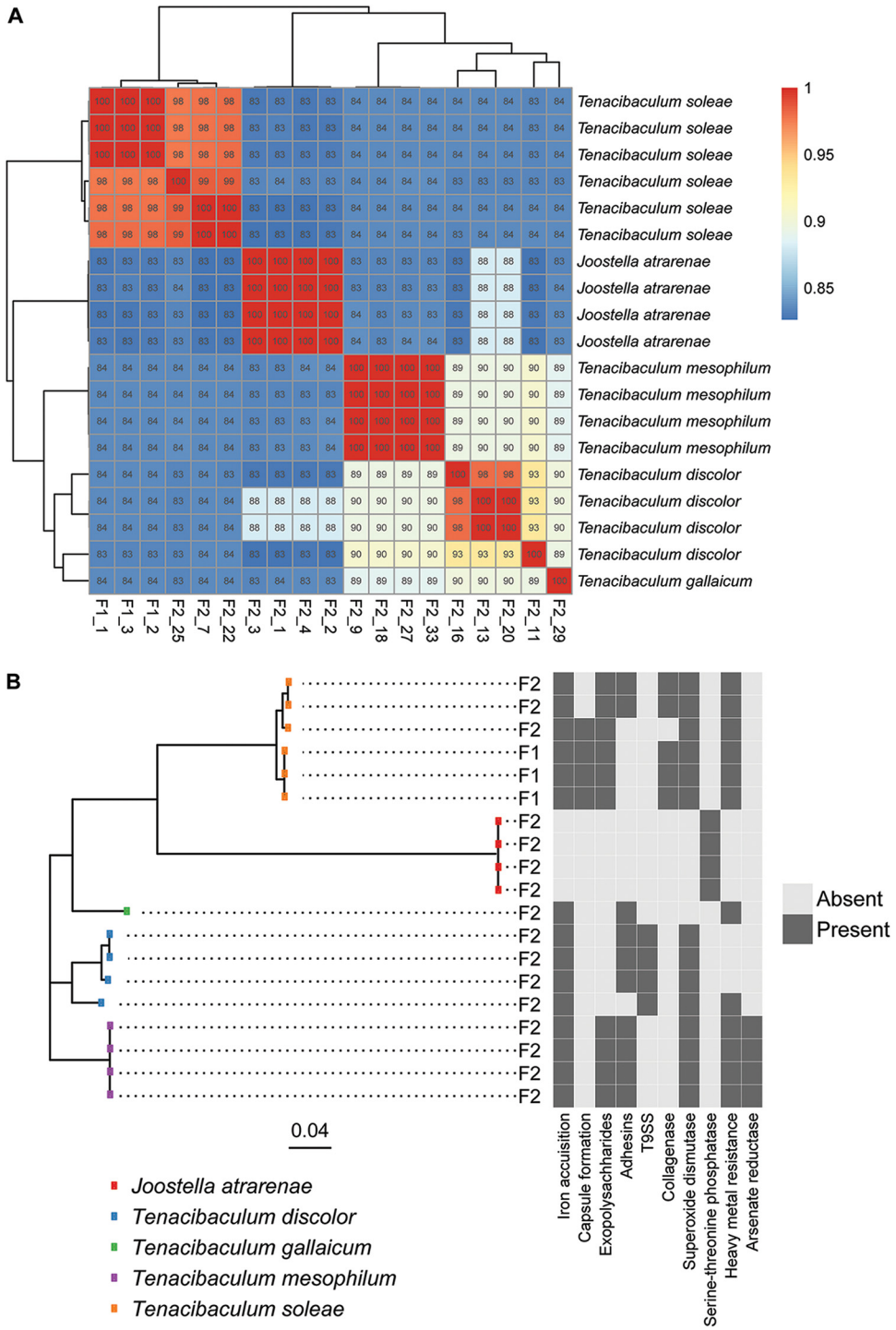

FIG 1 (A) Genomic heterogeneity among the 19 flavobacterial genomes depicted as the average nucleotide identity (ANI). The assigned taxonomy on the right is based on 16S rRNA gene sequence similarity. (B) Phylogenetic tree based on the alignment of 411 concatenated core genes with the virulence factor profile (a minimum of one gene per category) of the individual isolates on the right. F1 and F2 refers to the two aquaculture facilities, and the scale bar represents substitutions per nucleotide.

included *Tenacibaculum* type strains, were as efficient in forming biofilm on abiotic surfaces as the potential probiotic roseobacter control strain *Phaeobacter inhibens* DSM 17395, which exhibited significantly higher optical density at 590 nm (OD_{590}) values (one-way analysis of variance [ANOVA], Tukey's *post hoc*, $P < 0.05$; Fig. 2).

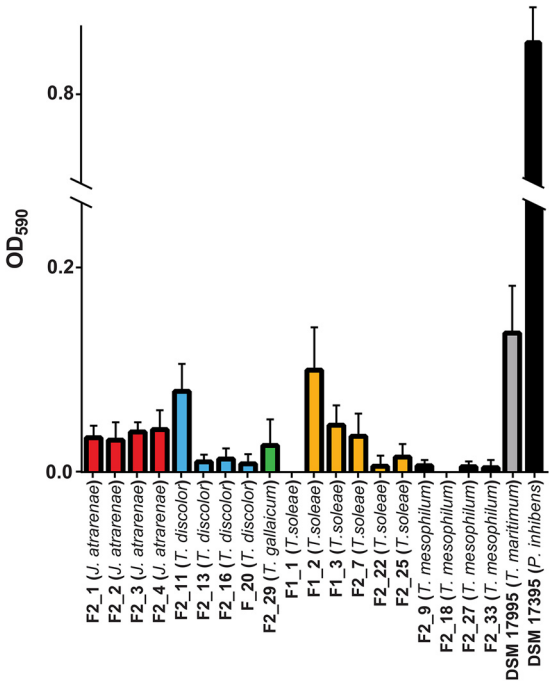

FIG 2 Biofilm formation by the 19 flavobacterial isolates, the *Tenacibaculum* type strains *T. maritimum* DSM 17995 and *T. mesophilum* DSM 17364, and the roseobacter *Phaeobacter inhibens* DSM 17395 (positive control). The OD_{590} values from the negative control (MB medium) were subtracted from the measurements. Error bars represent the standard deviations between biological triplicates.

Killing of flavobacterial pathogens by *Phaeobacter piscinae* S26. TDA-producing roseobacters are proficient biofilm formers on biotic and abiotic surfaces, and the production of TDA (per cell) is enhanced at the biofilm stage (37). We therefore envision that the roseobacters will be introduced, e.g., as biofilm on algal cultures that are used as live feed in marine fish larval rearing. We therefore applied a combined surface-liquid system in the investigation of the potential antagonism of a TDA-producing *Phaeobacter piscinae* S26 (38) against selected representatives of the flavobacterial isolates, as well as the type strain *T. maritimum* DSM 17995. *P. piscinae* S26 biofilms were established on the pegs of minimum biofilm eradication concentration (MBEC) assay plates with a density of $\log_{10}$ 6.52 $\pm$ 0.147 CFU peg^{-1}. Subsequently, planktonic cultures of *T. soleae* F1_2, *J. atrarenae* F2_1, *T. mesophilum* F2_9, *T. discolor* F2_11, *T. gallaicum* F2_29, and *T. maritimum* DSM 17995 were exposed to pegs colonized with S26 for 4 days. With this approach, substantial killing ($>$1 $\log_{10}$-fold reduction) of flavobacterial strains was observed for four of the six tested strains and, as for biofilm formation, susceptibility to the probiotic treatment was highly species specific. The pathogenic *T. maritimum* DSM 17995 type strain was the most susceptible, exhibiting a $>$5-fold $\log_{10}$ reduction compared to the untreated control ($P < 0.001$; Table 1). For *T. gallaicum* F2_29 and *T. mesophilum* F2_9, little to no effect of the treatment was observed, whereas pronounced inhibition was observed for *Tenacibaculum soleae* F1_2, *J. atrarenae* F2_1, and *T. discolor* F2_11 with abundances 1.78 to 2.44 orders of magnitude lower in the presence of the probiotic strain. The observed differences in efficacy of the treatment were due to variations in TDA sensitivity between flavobacterial species as sizeable zones of inhibition (ZOIs) were observed when the TDA-producing *P. piscinae* S26 and *P. inhibens* DSM 17395 strains were spotted onto lawns of *T. soleae* F1_2, *J. atrarenae* F2_1, *T. discolor* F2_11, and *T. maritimum* DSM 17995, whereas little to no ZOIs were observed on *T. mesophilum* F2_9 and *T. gallaicum* F2_29 lawns. The ZOIs observed were similar to—and in the case of *T. maritimum* DSM 17995 larger than—the zones produced on the highly susceptible control strain *Vibrio anguillarum* 90-11-286. ZOIs were not observed when spotting a *P. inhibens* DSM 17395 Δ*tdaB* mutant

TABLE 1 Killing of representative flavobacterial isolates and the *T. maritimum* DSM 17995 type strain by *Phaeobacter piscinae* 26 biofilms

Strain (species)	Mean ± SD			
	Log_{10} CFU mL^{-1} after exposure to S26	Control log_{10} CFU mL^{-1}	Log_{10} reduction	*P*[a]
F1_2 (*T. soleae*)	6.17 ± 0.28	8.45 ± 0.04	2.28	0.031
F2_1 (*J. atrarenae*)	6.83 ± 0.38	9.27 ± 0.03	2.44	0.0018
F2_9 (*T. mesophilum*)	8.39 ± 0.04	9.04 ± 0.08	0.65	0.017
F2_11 (*T. discolor*)	7.24 ± 0.20	9.02 ± 0.13	1.78	<0.001
F2_29 (*T. gallaicum*)	8.84 ± 0.30	8.63 ± 0.23	–0.21	0.39
DSM 17995 (*T. maritimum*)	<3[b]	8.40 ± 0.14	>5.40	<0.001

[a]As determined by a paired *t* test.
[b]Below the detection limit of 3.

against any of the strains (Fig. 3). No homologs to the known TDA resistance genes present in the genomes of TDA-producing roseobacters were found in the genomes of the flavobacterial isolates, and hence these strains draw on alternative means to evade the antibacterial effects of TDA.

While TDA is known to interfere with ATP generation through disruption of the proton motive force in *E. coli* (31), gene expression data suggest that TDA may have multiple targets in marine fish- and human-pathogenic *Vibrio vulnificus* (32). To investigate whether the observed divergence in susceptibility among species and strains could be explained by the presence or absence of specific genes, per-gene Fisher exact tests using Scoary were performed using ZOIs as a proxy for susceptibility. The observed ZOIs were significantly associated with the presence of a total of 219 annotated consensus genes and the absence of nine genes (see Table S2). Among others, the presence of genes involved in putative drug efflux, electron transport and ATP generation, universal stress, oxidative stress, metal ion stress, and iron acquisition were identified as being significantly associated with TDA tolerance (Fig. 4). Species exhibiting mean ZOIs of ≤1 mm all possessed three putative drug efflux-encoding genes, i.e., the TolC-related tripartite efflux-encoding gene *mdtE* and the efflux pump membrane trans-

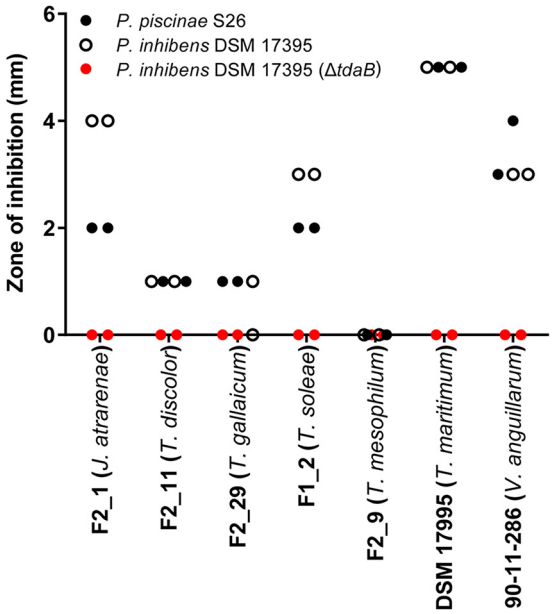

FIG 3 Zones of inhibition around roseobacter colonies spotted onto lawns of *Joostella atrarenae* F2_1, *Tenacibaculum discolor* F2_11, *Tenacibaculum gallaicum* F2_29, *Tenacibaculum soleae* F1_2, *Tenacibaculum mesophilum* F2_9, the *Tenacibaculum maritimum* type strain DSM 17955, and the TDA-sensitive *Vibrio anguillarum* 90-11-286. Zones were measured from the outside of the spot to correct for differences in roseobacter colony sizes on duplicate plates.

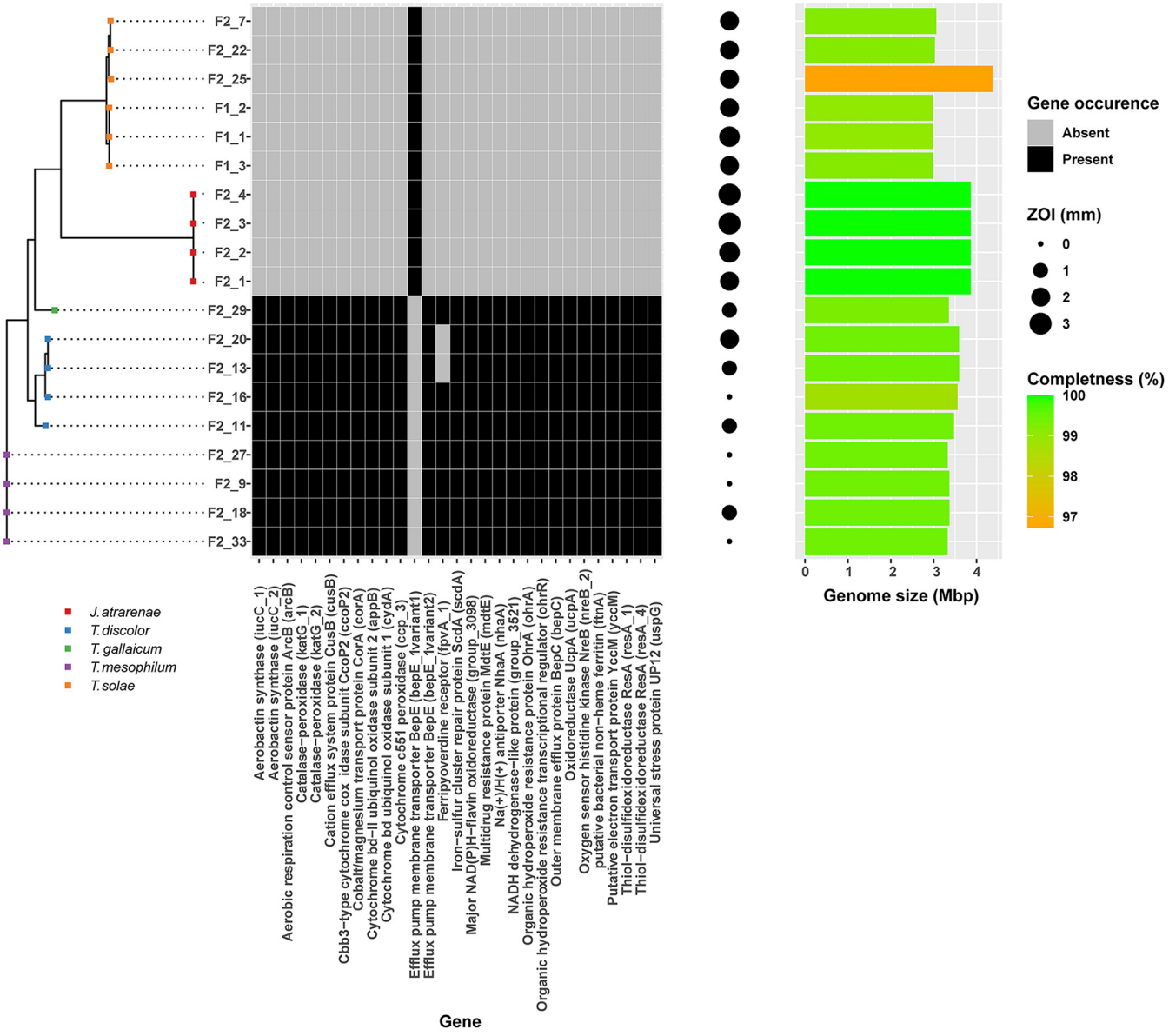

FIG 4 Gene occurrence profile of selected genes significantly associated with tolerance toward TDA in the 19 flavobacterial isolates. The sizes of the zones of inhibition are used as proxies for TDA susceptibility in the analysis. The genome completeness and size of the assembled genomes are presented as a bar chart on the right.

porter-encoding genes *bepC* and *bepE*, which were absent in susceptible species (mean ZOIs $>$ 1 mm), with the exception of F2_20, which was the only *T. discolor* strain exhibiting some susceptibility. Generally, susceptible strains harbored a divergent homolog to one of the efflux genes, *bepE*, yet it exhibited $<$70% sequence similarity to the gene found in tolerant strains. In addition, tolerant species possessed the gene encoding the Na^+/H^+ antiporter, NhaA, which facilitates the exchange of protons for sodium ions across the membrane. The same species harbored a series of genes related to electron transport, including the putative electron transport protein YccM, several cytochrome oxidases, oxidoreductases, and regulatory genes involved in oxygen sensing and respiration control (Fig. 4). Except for *T. discolor* F2_20, multiple stress-related genes were observed in species exhibiting tolerance toward TDA, including a universal stress protein-encoding gene homologous to *uspG* in *E. coli*, which is involved in regulation of cell motility in response to starvation, heat shock, and exposure to toxic substances. Furthermore, genes related to oxidative stress (peroxidase-

encoding genes) and metal ion stress/efflux (*cusB* and *corA*) associated with TDA tolerance. In contrast to the susceptible strains, tolerant isolates also carried the capacity to synthesize a putative aerobactin-like siderophore and the iron storage protein ferritin. However, confirmation of the ability to produce siderophores under the tested laboratory conditions was not possible for any of the strains (see Table S3). All aforementioned traits were conserved at the species level; however, the *T. discolor* isolates represented more pronounced overall genomic heterogeneity within the species (Fig. 1), which also came across as a relatively high degree of variation in the ZOI sizes among isolates (x- = 1 $\pm$ 0.7 mm). Two *T. discolor* isolates exhibiting smaller ZOIs of 0 mm (F2_16) and 1 mm (F2_11) both harbored a gene homologous to a ferripyoverdine receptor, while the other two isolates did not (F2_20; 2 mm ZOI, F2_13; 1 mm ZOI).

DISCUSSION

Cost-efficient rearing of fish at high densities is challenged by outbreaks of opportunistic pathogenic bacteria. To mitigate this issue while ensuring sustainability, probiotics have been suggested as an alternative to the prophylactic use of antibiotics to control pathogens and prevent disease outbreaks (2, 28). Currently, little is known about the virulence factors of the emerging broad-host-range pathogenic flavobacterial genus *Tenacibaculum*, let alone its susceptibility to probiotic treatment, and the results reported here demonstrate that outbreaks of flavobacterial fish pathogens may involve heterogeneous assemblages of strains that likely draw upon multiple different virulence factors. Furthermore, these diverse pathogenic strains exhibit different levels of susceptibility to the promising TDA-producing probiotic candidate *P. piscinae* S26 (28, 29). However, several strains were highly sensitive, indicating that TDA-producing roseobacters could hold potential in control of tenacibaculosis.

The 15 *Tenacibaculum* isolates obtained were distributed among four different species, all previously shown to be associated with disease in cultured finfish (5, 39–41). At F1, only three clonal isolates were recovered, whereas the outbreak at F2 exhibited significant heterogeneity within the genus and within *T. discolor* isolates, corroborating the notion, that such local *Tenacibaculum* epidemics can involve multiple different strains, hence complicating efforts to produce effective vaccines (42). Interestingly, four clonal isolates of a closely related genus, *Joostella*, was also recovered from F2, although isolates of this genus have previously only been recovered from environmental sources (43, 44) and has to our knowledge not been associated with disease outbreaks in aquaculture. To what extent these bacteria are actively involved in infection remains to be determined, but the four *Joostella* strains did carry the genetic capacity to produce a single, though unique potential virulence factor, namely, an STP, which have been reported to be important for the regulation of virulence expression and survival within infected mammalian hosts (45, 46).

Within the genus *Tenacibaculum*, fish-pathogenic lineages are dispersed among lineages of environmental strains across clades, suggesting parallel evolution of traits involved in pathogenicity (39). While all 15 *Tenacibaculum* strains in this study possessed genetic determinants relevant for iron acquisition, our results corroborate the observation of parallel evolution as no single virulence gene was conserved across all 15 genomes. In addition to iron acquisition factors, which may be broadly employed as a means to sequester iron from host proteins in flavobacterial fish pathogens (47), mechanisms to evade the reactive oxygen species (ROS) from host macrophages were widely distributed among the isolates as well. This suggests that mechanisms involved in survival within the host are essential and similar across *Tenacibaculum* species, while the mechanisms by which they gain entry into the host, e.g., through adhesion, collagenase production, or secretion systems, deviate.

Tenacibaculosis is generally assumed to be preceded by biofilm formation on skin and gills (14). *T. maritimum* has recently been shown to form biofilms on inert surfaces as well, allowing it to persist in the aquaculture environment (48). We observed biofilm formation by *T. maritimum* DSM 17995 and a subset of the *T. soleae* and *T. discolor* strains on inert surfaces. However, the TDA-producing roseobacter *P. inhibens* DSM 17395 control strain was much more proficient in biofilm formation. The closely related

species *Phaeobacter gallaeciensis* 2.10 is capable of successfully invading and dominating marine epiphytic biofilms (49), and as we imagine probiotic TDA-producing *Phaeobacter* spp. (49), being administered as biofilms on live feed organisms, or as biofilter constituents (2), the pronounced difference in biofilm production between the pathogens and the roseobacter is a promising observation. Pursuing this line of thinking further, we investigated the ability of pregrown biofilms of the indigenous aquaculture probiotic *P. piscinae* S26 (29) to kill selected representative pathogenic strains.

While TDA can inhibit or kill a wide range of Gram-positive and Gram-negative bacteria, including fish and human pathogens (28, 29, 33, 50–55), a high number of naturally tolerant strains have been observed in marine microbial communities. Furthermore, the impact of TDA or TDA-producing microorganisms on microbiomes associated with eukaryotes, including aquaculture relevant live feed organisms, fish eggs, and larvae, is subtle and may only affect specific species within a genus (36, 56). Accordingly, we observed widely different killing effects of *P. piscinae* S26 biofilms on the different *Tenacibaculum* species. The fish pathogenic type strain *T. maritimum* DSM 17995, and the *T. soleae*, and *T. discolor* representative strains were efficiently killed, whereas the selected *T. mesophilum* and *T. gallaicum* strains were largely unaffected. We could confirm that tolerance toward TDA was the cause of the differential killing efficacy observed between the species, so TDA-producing probiotics may only provide efficient protection against a subset of *Tenacibaculum* pathogens. Still, more extensive testing in complex nonaxenic live feed systems and challenge trials are needed to ascertain the efficacy of *P. piscinae* S26 against these pathogens *in situ*.

TDA tolerance in *T. discolor* and *T. mesophilum* was not associated with identified TDA resistance genes (*tdaR1-3* [31]) but rather with hitherto-undescribed mechanisms. We identified the presence of no less than 228 annotated genes, which were significantly associated with tolerance, and yet the tolerant phenotype also followed the overall phylogenetic relationship between strains; hence, causality could not be inferred directly from our analysis. Nonetheless, we did observe that the presence of a multidrug efflux pump was significantly associated with tolerance and similar systems have previously been demonstrated to confer drug resistance in fish pathogenic flavobacteria (57). Hence, chromosomally encoded efflux systems may be important for the efficacy of TDA against *Tenacibaculum* pathogens.

TDA inhibits target bacteria through acidification of the cytosol by import of protons and concurrent export of metal ions. As a result, the proton motive force is disrupted, ATP is depleted, and cells are killed (31). In the human and fish pathogen *Vibrio vulnificus*, the effects of TDA exposure results in reduced expression of the final parts of the electron transport chain (32), likely resulting in energy depletion and accumulation of intracellular superoxide radicals (58). The tolerant strains all harbored 15 genes involved in oxidative phosphorylation, in its regulation, and in oxidative stress relief. These genes were absent in susceptible strains and, accordingly, differences in TDA susceptibility across the *Tenacibaculum* genus may be a result of inherent differences in their physiology, specifically pertaining to energy generation and stress responses.

TDA has weak iron chelating properties, and yet its production in laboratory cultures is dependent on the presence of iron. Hence, iron sequestration is likely not the main function of TDA (59). However, when exposed to subinhibitory concentrations of TDA, *V. vulnificus* responds by upregulating the expression of genes involved in iron sequestration, including siderophore-encoding genes (32). Our findings substantiate the observation that iron is important for the efficacy of TDA since we found the tolerant strains to possess genetic determinants involved in iron sequestration and storage, e.g., through putative siderophores and siderophore receptors. Within the heterogeneous group of *T. discolor* isolates, the two strains exhibiting highest tolerance to TDA also possessed a ferripyoverdine receptor not recovered in the genomes of the two more susceptible strains.

In conclusion, local outbreaks of flavobacterial fish pathogens may involve heterogeneous groups of strains with the genetic capacity to produce multiple different virulence factors related to host invasion but with largely similar mechanisms of host immune evasion. The probiotic *P. piscinae* S26 is promising against *Tenacibaculum* pathogens such as

T. maritimum and *T. soleae*. However, it may not prevent tenacibaculosis altogether, since strains of the *T. discolor* species, as well as the *T. gallaicum* and *T. mesophilum* species, exhibit a natural tolerance toward TDA. While we cannot determine which of the specific traits identified to correlate with tolerance are directly causative, the data suggest that multiple inherent physiological features contribute to TDA tolerance in concert, likely including multidrug efflux systems, electron and proton transport mechanisms, stress response mechanisms, and features involved in iron sequestration and storage.

MATERIALS AND METHODS

Strains and media. Except for the type strains *T. maritimum* DSM 17995, which was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ), and the *Vibrio anguillarum* 90-11-286 control strain (60, 61), all pathogenic strains were supplied by aquaculture facilities (F1 and F2). *Roseobacter* group bacteria used in this study included *P. piscinae* S26 originally isolated from F2 (29, 38), as well as *P. inhibens* DSM 17395 and its corresponding TDA-deficient mutant (62). All bacteria were grown on marine agar (MA) or in marine broth (MB; Difco 2216) at 25°C for 48 h (with shaking at 200 rpm) unless otherwise stated.

16S rRNA gene sequencing. For identification of bacterial isolates, colony mass from a single colony was transferred to 50 μL of 0.05 M NaOH in 1.5-mL microcentrifuge tubes. Tubes were incubated at 100°C for 10 min and centrifuged at 11,000 $\times$ *g* for 120 s. One microliter of supernatant was used as the template in a 25-μL PCR containing 2.5 μL of 10 $\times$ Hot Start PCR buffer, 2.5 μL of 2 mM dNTPs, 0.8 μL of 12.5 μM 27F primer, 0.8 μL of 12.5 μM 1492R primer (63), 4 μL of 25 mM $MgCl_2$, and 0.125 μL of Maxima Hot Start polymerase in ddH_2O (63). The reaction was performed in a Bio-Rad T100 thermal cycler as follows: 1 cycle at 95°C for 300 s; followed by 35 cycles at 95°C for 45 s, 51°C for 60 s, and 72°C for 120 s; followed finally by a cycle at 72°C for 420 s. The PCR product was cleaned by the addition of 0.5 μL of Exonuclease I (EN0581; Thermo Scientific) and 0.5 μL of Fast AP (EF0651, Thermo Scientific), followed by consecutive 15-min incubations at 37°C and 85°C. Amplicons were sequenced at Macrogen Europe (Amsterdam, Netherlands), and forward and reverse reads were trimmed (quality score of 0.05) and assembled (minimum aligned length, 20 nucleotides) using CLC Main Workbench 8.1 (Qiagen Bioinformatics). Some sequences could not be assembled using these criteria, and in these cases the forward and reverse reads were analyzed separately (see Table S1). Identities were assigned by BLASTn against the NCBI database.

DNA extraction and whole-genome sequencing of flavobacteria. Cells from 2 mL of liquid culture were pelleted by centrifugation at 5,000 $\times$ *g* for 5 min, and DNA was extracted using a NucleoSpin tissue kit (Macherey-Nagel) according to the manufacturer's instructions, using 10 mM Tris-HCl (pH 8.5) as the elution buffer. DNA concentrations and purity were determined using a Qubit 2.0 fluorometer (Invitrogen) and a DS-11+ NanoDrop spectrophotometer (DeNovix), respectively. Paired-end (2 $\times$ 300 PE) sequencing was performed using an Illumina MiSeq platform (V3 chemistry), aiming at a 100$\times$ coverage. *De novo* assembly was performed with SPAdes 3.13.0 using a Shovill pipeline (64, 65). 16S rRNA genes were extracted with Barrnap and aligned to the NCBI database for species verification (66), and the average nucleotide identity (ANI) between the genomes was calculated with MUMmer 3.0 using the Pyani module (67, 68). Genomes were compared to all type strain genomes available in the TYGS database via the MASH algorithm (69) to confirm 16S species verification where possible. Using BLASTn, the genomes were aligned to the "VFDB" virulence factor database (VFDB, 2019), as well as to a custom-made database consisting of the *tdaR1-tdaR3* genes encoding the TDA resistance factors in *P. inhibens* (31), and virulence genes were identified in *T. maritimum* DSM 17995 (13) and *F. psychrophilum* (70). Homologs were determined as open reading frames with a minimum sequence similarity of 70% and a minimum length of 30 bp. Genome sizes were determined using Quast, including contigs of >1,000 bp, and genome completeness was estimated with Busco v4.1.4 using the lineage data set flavobacteriales_odb10 (71, 72).

Core genes were identified and aligned using Roary with a blastp cutoff of 70% and if they were present in at least 95% of the sequence isolates (73). The core genes at a nucleotide level were then aligned with MAFFT, and the resulting alignment was used to generate a phylogenetic tree using Fasttree (74).

Biofilm formation. The ability of the isolates to produce biofilms on abiotic surfaces was determined using an O'Toole-Kolter assay (75) with modifications as described elsewhere (76). All flavobacterial isolates, the type strains *T. maritimum* DSM 17995 and *T. mesophilum* DSM 13764, and the positive-control *P. inhibens* DSM 17395 were grown in 10 mL of MB at 25°C overnight. All bacterial cultures were diluted to an OD_{600} of 0.01, and 100 μL was transferred in triplicate to a 96-well microtiter plate (Thermo Scientific Nucleon Delta Surface). Sterile medium was used for negative controls. Subsequently, the plate was incubated for 21 h at 25°C, followed by 10 s of shaking. The OD_{600} was measured using a SpectraMax i3 spectrophotometer. Next, the liquid was removed, the wells were washed three times with 100 μL of dH_2O, and the plate was left to dry for 5 min. After drying, 125 μL of a 1% crystal violet (Sigma-Aldrich) solution was added to each well, followed by incubation for 15 min. The crystal violet solution was removed, and the wells were washed three times with 200 μL of dH_2O. After the wells were dried for 15 min, 200 μL of 96% ethanol was added, and the plate was incubated for 30 min. Then, 100 μL of the ethanol-crystal violet mixture was added to a new microtiter plate. The plate was shaken for 10 s, followed by measurement of the optical density (OD_{590}). To determine significant differences in biofilm formation by the pathogenic species compared to the probiotic *P. inhibens* DSM 17395 positive control, one-way ANOVA, followed by Tukey's test, was performed.

Inhibition of pathogens by *P. piscinae* S26 biofilms. The assay was performed following procedures described by D'Alvise et al. (77). Briefly, *P. piscinae* S26 was incubated in 10 mL of MB overnight at

25°C under stagnant growth conditions. Subsequently, the culture was diluted 40-fold in 200 μL of MB in wells of a 96-well Innovotech MBEC P&G assay plate. The plate was incubated stagnant for 4 days at 25°C. After incubation, duplicate pegs were removed and transferred to 2 mL of 3% Instant Ocean (Instant Ocean sea salts; AquariumSystems, Inc., Sarrebourg, France) and sonicated for 4 min at 28 kHz, followed by vortexing for 30 s to detach bacterial cells from the peg surfaces. A dilution series was plated on MA plates for enumeration. After the removal of excess liquid on the remaining pegs (not sonicated, vortexed) carrying *P. piscinae* S26 biofilms, the pegs were transferred directly to wells containing MB cultures of the strains F1_2, F2_1, F2_9, F2_11, F2_29, and *T. maritimum* DSM 17995 in triplicates. Sterile pegs and MB were used for negative controls. The MBEC plates were incubated for 4 days at 25°C under stagnant conditions after which bacterial abundances were determined on MA plates supplemented with 25 μg mL^{-1} streptomycin. Colony counts were log transformed, and significant changes were determined using F tests for variance, followed by two-tailed *t* tests ($\alpha = 0.05$).

TDA susceptibility. An agar diffusion (spot inhibition) assay was performed to determine the sensitivity of F1_2, F2_1, F2_9, F2_11, F2_29, and *T. maritimum* DSM 17995 toward TDA. The optical densities of flavobacterial cultures were adjusted to an OD_{600} of 0.5, and a sterile cotton swab was used to plate culture material onto MA plates to form bacterial lawns. Then, 5 μL of ON culture of the two wild-type TDA producers and the TDA-deficient mutant were spotted onto the surfaces of the lawns in duplicates. The TDA-sensitive *V. anguillarum* 90-11-286 strain was included as a positive control. ZOIs were measured from the edge of the spot to adjust for the slight variations in roseobacter colony sizes.

Associations of TDA susceptibility and gene occurrence. Tolerance was assessed with an approach identical to the TDA susceptibility test described above, including all the flavobacterial isolates, but only including *P. piscinae S26* as the spotted culture. The resulting ZOIs were used as a proxy for TDA susceptibility. A gene presence/absence matrix was generated based on the *de novo*-assembled genomes using Roary v3.13.0 with a minimum blastp percentage of 70% (73). Scoary v.1.6.16 was then run on the gene presence absence matrix, and the ZOIs were taken as a trait, using a cutoff of $>$1 mm (78). Genes with a Bonferroni-corrected *P* value of $<$0.05 in the Fisher exact test in Scoary were deemed significantly associated and annotated.

Determination of siderophore production. Precultures of the representative strains F1_2, F2_1, F2_9, F2_11, F2_29, the type strain *T. maritimum* DSM 17995, and a siderophore-producing positive-control strain, *Pseudoalteromonas piscicida* S2040 (79), were diluted 100-fold into triplicate tubes of three different liquid growth substrates: 1.5% $SS_{CAS+GLU}$ (1.5% Sigma Sea Salts, 0.3% Casamino Acids, 0.4% glucose) 1.5% $SS_{CAS+MAN}$ (1.5% Sigma Sea Salts, 0.3% Casamino Acids, 0.4% mannose), and 1/2YTSS (2 g of Bacto yeast extract, 1.25 g of Bacto tryptone, 20 g of Sigma Sea Salts L^{-1}). The cultures were incubated for 7 days at 15°C and 25°C, and sterile-filtered supernatant was subsequently mixed 1:1 with CAS solution (80). The assay reaction was inspected at 2 h, 24 h, and 5 days. Positive reactions were only observed for the positive-control strain, and the growth of the *Tenacibaculum* strains in the substrates was limited overall.

Data availability. Partial 16S rRNA gene sequences obtained from the isolates were deposited at the National Center for Biotechnology Information (NCBI) under the accession numbers MN481000 to MN481046, and whole-genome sequencing reads were deposited in the Sequencing Read Archive at the NCBI under accession number PRJNA566079.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank Jette Melchiorsen for technical assistance.

This study was supported by the Danish National Research Foundation (DNRF137) and by a grant from The Danish Council for Strategic Research Program Commission on Health, Food, and Welfare (12-132390; ProAqua).

We declare there are no conflicts of interests.

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