Absence of *Staphylococcus aureus* in wild populations of fish supports a spillover hypothesis

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Abstract

*Staphylococcus aureus* is a human commensal and opportunistic pathogen that can also colonise and cause disease in other animal species. In humans and livestock, where *S. aureus* is most studied, there is evidence that strains have different host specialisms. Recent studies have found *S. aureus* in many wild animals, including fish, whose physiologies and ecologies are very different to humans. However, it remains unclear whether *S. aureus* is adapted to and persisting within these species, or if its presence is due to repeated spillover from a source population. Distinguishing between these two scenarios is important for both public health and conservation. In this study we looked for evidence to support the hypothesis that the presence of *S. aureus* in fish is the result of spillover, through testing for the presence of *S. aureus* in fish that are isolated from likely source populations. We sampled 123 brown trout and their environment from 16 sites in the Scottish Highlands. All these sites are remote and have very low populations density of wild animal species known to carry *S. aureus*, but were selected to represent variable levels of exposure to humans, avian and livestock species. While our sampling methods readily detected *S. aureus* from the external and internal organs of a farmed fish, we did not detect *S. aureus* in any wild trout or their environment from any of the 16 sites. We sequenced 12 *S. aureus* isolates from the farmed fish. While they were all from clonal-complex 45, the genomic diversity was high enough to indicate repeated acquisition from a source population. In addition, the presence of a φSa3 prophage containing a human immune evasion cluster indicates a recent history of these isolates within human populations. Taken together, our results support the presence of *S. aureus* in fish being due to spillover from other host populations, rather than the adaptation of *S. aureus* to aquaculture or fish populations. Given predictions that fish consumption will increase, more whole genome sequencing of *S. aureus* in aquaculture is needed to understand the presence of *S. aureus* in these environments and to mitigate the risk to fish and human health.
Introduction

*Staphylococcus aureus* is both a common commensal bacterium of the human nasopharynx and skin, and an opportunistic pathogen (1). *S. aureus* also colonizes and causes infections in companion animals (2,3) and livestock (4,5) with the latter resulting in significant morbidity and economic loss (6). Recent studies have shown that major human and livestock strains, which are distinguished by a profile of housekeeping genes and named clonal complexes (CC), are also found in many wild animal species (7-12). However, little is known about whether this is a consequence of recent spillover from human and/or livestock populations (i.e., limited transmission of a strain in a novel host group with no evidence of adaptation), or if they persist independently within wild animal species. These two scenarios have different implications for both public health and conservation. For spillover, human and livestock are major sources of environmental contamination with a bacterium that is capable of causing disease in diverse host species, and often resistant to antibiotics. Persistent carriage or infection of novel emergent strains of *S. aureus* in wild animal species may pose a public health risk to human and livestock health (13-16). To distinguish between these two possibilities, genomic data from isolates from wild animal populations can be used to infer transmission dates and routes, and to identify genetic changes that may represent host-specific adaptation (5, 17-25).

*S. aureus*, including methicillin resistant *S. aureus* (MRSA), is frequently reported in fish and fishery products, with prevalence ranging from 2-60% (26). The presence of *S. aureus* in fish is concerning because the ingestion of staphylococcal enterotoxins produced by *S. aureus*, can cause food poisoning (27,28). It is widely assumed that *S. aureus* in fish products is a result of contamination during handling and processing (29). However, the combined use of antimicrobial drugs in aquaculture and subsequent contamination of aquatic environments, could contribute to the selection, emergence and spread of antibiotic resistant *S. aureus* (30,31). Supporting this conjecture, *S. aureus* has also been found in fish sampled directly from aquaculture, where fish processing and handling is limited and MRSA have been identified in cage-cultured tilapia in Malaysia (32), tank cultured dusky kob in South Africa (33), and farmed fish in Iran (34). Only one study by Salgueiro *et al.* (2020), performed genome sequencing and found CC398 methicillin-sensitive *S. aureus* (MSSA) (35), most likely human-associated (22,36,37). This study suggests that *S. aureus* is present in fish due to spillover from human populations but does not have the discrimination to test whether there is adaptation and persistence of *S. aureus* within fish populations or aquatic environments.

In this study, we investigate whether *S. aureus* is present in fish from potential source populations in the Scottish Highlands. The Scottish Highlands are among the least densely populated regions of Europe at an average density of 8 persons per km² (38). We sample wild brown trout (*Salmo trutta* L.1758) and their environment, including water and sediment. Brown trout vary in habitat, ranging from highland streams to arctic fjords (39), which allows us to investigate the prevalence of *S. aureus* in areas with varied levels of exposure to known hosts of *S. aureus*. We sample from lochs close to livestock, lochs within bird roosting/feeding areas, sea sites close to human populations, and remote lochs that are isolated from human, bird, and livestock populations. While we readily detected *S. aureus* from multiple organs of a farmed fish in London, we did not detect *S. aureus* in any Scottish trout, nor their environment.
These results support the presence of *S. aureus* in farmed fish being due to recent spillover from other host populations.

**Methods**

**Calibration of *S. aureus* isolation from fish**

To optimise our method of isolation of *S. aureus* from fish, a pilot experiment was carried out on a single rainbow trout (*Oncorhynchus mykiss*) purchased from a fish farm in London, England. Wearing gloves, we swabbed the mouth, vent, gill, and skin with charcoal swabs (Medical Wire Transwabs®), and then inoculated into 10ml of 6% NaCl *Staphylococcus* selective media (A&E Laboratories). We dissected and swabbed the intestine, gill, skin, and heart, and transferred each organ to a sealed processing bag containing 10ml 6% NaCl *Staphylococcus*-selective media, homogenising with a Stomacher (Stomacher80 Laboratory System, Seward Ltd, UK). We took nasal swabs from both researchers carrying out sample processing to control for potential contamination, processed in the same way as fish swabs. We incubated all samples at 37°C in universal tubes for 24h. After incubation, we plated 10-100μL of each culture (depending on the culture cloudiness) onto Brilliance Staph 24 Agar plates (Oxoid, UK) and incubated at 37°C for 24h. For each positive plate, we confirmed *S. aureus* by selecting three colonies for a *femB* PCR (see PCR protocol below).

**Polymerase Chain Reaction (PCR) to amplify *femB***

To confirm the presence of *S. aureus*, we used a colony PCR with *femB* primers. The primers were FemB1 (5′-CAT GGT TAC GAG CAT CAT GG) and FemB2 (5′-AAC GCC AGA AGC AAG GTT TA), leading to an *S. aureus*-specific 447bp PCR product (41). We touched a pipette tip on a single *S. aureus* colony and mixed it with 20 μl of water in a PCR tube. We boiled the samples for 5 min at 95°C. Each sample contained 2μl of boiled cell solution and 18μl of the PCR master mix with MyTaq DNA Polymerase (Bioline). The PCR cycling conditions were 95 °C 5 min, 30 cycles (95 °C-15 s, 58°C – 10s, 72°C 30s), 72°C for 10 min. We added 2.5μL Sybr® Safe DNA gel stain (Thermo Fisher, UK) to 100μL agarose solution, which was 1% w/v agarose dissolved by heating in a 1×TBE buffer (Tris-borate-EDTA). We loaded 15μL of PCR reaction mixture onto each well, with a 5μL 5 HyperLadderTM 100bp (Bioline) to confirm product size. Electrophoresis was performed in a Sigma-Aldrich electrophoresis tank with 1 ×TBE at 100V for 40 minutes. Electrophoresed gels were visualised under blue light, and their images visualised with the GelDocTM XR System Imager (BioRad).

**Sequencing**

For all confirmed and suspected *S. aureus* positive samples from the farmed fish, two colonies were selected for each sampling method (swabbing and tissue samples) per positive sampling site (gill, intestine, skin). Genomic DNA was extracted from overnight cultures grown in Tryptic Soy Broth (TSB) at 37°C with 200rpm shaking using the MasterPure Gram Positive DNA Purification Kit (Cambio, UK). Illumina library preparation and Hi-Seq sequencing were carried out as described in (41).

**Genome assembly and MLST typing**
Published genome sequence data from isolates of CC45 were downloaded from the European Nucleotide Archive (ENA) and subsampled to represent host and geographical diversity (Table S1). Sequence data from both newly sequenced and publicly available CC45 isolates were assembled using Spades v.3.12.0 (42). Adapters and low-quality reads were removed with Cutadapt v1.16 (43) and Sickle v1.33 (44) and screened for contamination using FastQ Screen v0.12.0 (45). We identified optimal k-mers based on average read lengths for each genome. All de novo assemblies were evaluated using QUAST v.5.0.1 (46) and reads were mapped back to de novo assemblies to investigate polymorphism (indicative of mixed cultures) using Bowtie2 v1.2.2 (47). All assemblies were of good quality (i.e., N50 <10,000, contigs smaller than 1kb contributing to >15% of the total assembly length, total assembly length outside of the median sequence length +/- one standard deviation, or >1500 polymorphic sites).

**Phylogenetic analyses and genome annotation**

Reference-mapped assemblies were generated using Bowtie2 v1.2.2, using the reference genome LGA251 (GenBank accession no. GCA_000237265.1) (47). All genomes had average coverage <50x or with >10% missing sites. Recombination was identified in the reference-mapped alignment using Gubbins v2.3.1 and recombinant sites were masked from phylogenetic analyses (48). Sites that had either recombination detected or missing data were excluded from phylogenetic analyses. Phylogenetic reconstruction was carried out for the reference-mapped alignment with RAxML (v8.2.4) using the GTR+Γ model and 1,000 bootstraps (49) and rooted using an isolate from CC398 (SRR445234). To investigate the diversity among closely related isolates, we examined the reference mapped alignment to identify single nucleotide polymorphisms (SNPs). The alignment after recombination stripping was uploaded to Geneious 2020.0.4 (https://www.geneious.com), and all regions that were within 100bp of large regions containing missing and regions containing SNP clusters > 5 SNPs located within 50bp were removed. Next, the SNPs were extracted, and an MSTree V2 was constructed using GrapeTree (50).

We identified antibiotic resistance genes using the Pathogenwatch AMR prediction module (Wellcome Sanger Institute), which uses BLASTn (51) with a cut-off of 75% coverage and 80–90% identity threshold (depending on the gene) against a S. aureus antimicrobial resistance database. Presence of Sa3 prophages was established through searching for genes in the human immune evasion gene cluster using BLASTn (51) with a cut-off of 90% coverage and 90% identity threshold. The query human immune evasion genes were extracted from a reference genome (assembly accession: GCA_900324385.1). Presence of enterotoxins was investigated through searching for relevant proteins from a database compiled by Merda and Felten et al., 2020, using tBLASTn cut-off of 80% coverage and 80% (52).

**Sample size and statistical analysis**

To guide sample size design, we calculated the probability of detecting one or more positive sample with varying levels of prevalence using the pbinom function within the software package R (53). We show that 30 fish samples give us a 95% probability of detecting a positive sample if the true prevalence is around 10% (Figure S1). Prevalence of S. aureus in fish varies but is typically greater than 10% (26, 33). Therefore, we chose to sample 30 fish from each of our four habitats. In total, 120 fish gives a 95% probability of detecting a positive if the true prevalence at all sites is
around 3%. We calculated 95% confidence intervals shown in Table 1 on our observed prevalence, using the *binom.test* function in R (53).

![Figure S1: The probability of detecting one or more positive *S. aureus* isolate from fish samples (y axis), given differences in the true prevalence (x axis). The coloured lines correspond to different numbers of fish samples. For example, 20 fish samples gives a 95% chance of detecting one or more positive *S. aureus* isolate if the true prevalence is around 0.14.](image)

**Fish and environmental sampling**

All sampling was carried out in July 2019. Brown trout were captured by fly-fishing and transferred, along with fresh loch/sea water, to a sterilised bucket. We swabbed the vents and gills whilst the trout was alive, with operators wearing sterile gloves to prevent cross-contamination. Fish larger than 250mm were released (a condition of the landowners). We euthanized fish smaller than 250mm via a blow to the back of the head with a sterilised priest, placed them in a specimen bag and stored them in an icebox until later transferring to a -80°C freezer, for long term storage. The sites were selected based on their isolation from (Isolated Lochs) or their proximity (<1 km) to human frequented sites, including camping sites (Human), livestock grazing sites (Livestock Lochs) and bird nesting sites (Bird Lochs). All habitat types, except for Bird Lochs, were represented by either two or three site clusters >21 km apart.

To test whether *S. aureus* was present in the environment, we collected water and sediment samples from 25 sites, including all sites where we sampled trout. Sampling was carried out with operators wearing sterile gloves to prevent cross-contamination. We took water (3.5 litres) from approximately 5-10 cm depth using sterile 1000ml and 500ml containers. We collected sediment (approximately 50g per location) to sterile containers using a sterilised plastic scoop from littoral areas that were a) > 150mm below the water surface; and b) undisturbed. We kept both water and sediment samples chilled at 4°C, until analysis. Samples were processed immediately upon
arrival at the laboratory. We took nasal swabs from both researchers carrying out sample processing to control for potential contamination.

S. aureus isolation from environmental samples

To isolate S. aureus from water, we filtered three aliquots of 1000 ml per water sample through 0.45μm 47mm white gridded mixed ester cellulose membranes (Merck, USA). Membranes were placed on 55 mm Baird-Parker with potassium tellurite enrichment agar plates and incubated at 37°C for 48 hours. To isolate S. aureus from sediment, we transferred three 10 g (wet weight) sediment samples (per location) to sterile 50 ml centrifuge tubes. We added 25 ml of 2x concentrated Baird-Parker with potassium tellurite enrichment medium to each sample. After vortexing for approximately 45 s, the supernatant from each sample was transferred to a new sterile tube and incubated until a black precipitate formed (up to seven days). After this, the samples were vortexed for 30s, and 100μl was plated on 100 mm Baird-Parker with potassium tellurite enrichment agar plates and incubated at 37°C for 48 hours. For both water and sediment samples, after incubation, we transferred three presumptive S. aureus colonies (black colonies) onto fresh Brilliance Staph 24 plates (Oxoid, UK) and incubated at 37°C for 24 hours. For each potential positive plate, we confirmed S. aureus by selecting three colonies for a femB PCR (see PCR protocol below).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out with the Vitek 2 system (AES software, bioMérieux, Marcy l’Étoile, France) according to the manufacturer’s instructions. The isolates were plated onto Columbia Blood Agar (Oxoid Deutschland) and incubated at 37°C for 18-24h. One colony was picked with a sterile swab and mixed in the saline by vortexing. The OD$_{600}$ was measured and adjusted to 0.5-0.63. The inoculum was prepared by transferring 280μl to 3ml saline. Next, each sample was loaded into a VITEK® 2 AST-GP80 card. Susceptibility cards were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints (54).

Results

S. aureus sample calibration and whole genome diversity from a farmed fish

To establish the most effective method for detecting S. aureus from fish, we swabbed and homogenised tissue from different body sites of a farmed Rainbow trout (Oncorhynchus mykiss). S. aureus was successfully isolated from all sites, apart from a heart tissue sample and a mouth swab (Table 2). All positive tissue samples were also detected using charcoal swabs, suggesting that tissue processing is unnecessary for S. aureus detection from positive sites. Both swabs from researchers carrying out the sample processing were negative for S. aureus.

Table 2: The detection of S. aureus from a single rainbow trout purchased from a fish farm in London, England.

<table>
<thead>
<tr>
<th>Sampling method</th>
<th>Sampling site</th>
<th>S. aureus detection using selective plates</th>
<th>PCR of the femB gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charcoal swab</td>
<td>Mouth</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gills</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Vent</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tissue</td>
<td>Intestine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gills</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
To investigate within fish diversity, whole-genome sequencing was carried out for 12 *S. aureus* isolated from the different body sites. All isolates were identified as a strain type (ST) 54, which is part of clonal complex (CC) 45. Figure 1 shows a grape plot from a core genome alignment mapped to the *S. aureus* reference genome, LGA251. The isolates are not clustered by sampling site (intestine, gill, and skin) or by sampling method (charcoal swabbing and tissue). The isolates are very closely related, with the maximum distance between two isolates being 12 single nucleotide polymorphisms (SNPs). Based on an SNP clock rate of ~3.5 SNPs/genome/year in ST22 (55), we estimated that these isolates’ most recent common ancestor would have dated to around 2017 (two years before sampling).

To place the farmed rainbow trout isolates within the known diversity of CC45, we assembled the genomes from publicly available sequencing data from a set of CC45 isolates (Table S1). CC45 was mostly associated with human hosts (71%) but also identified in non-human hosts. The phylogeny of CC45 shows that all of the samples we collected from the farmed trout fall in a single clade, with a human *S. aureus* from the UK being the closest relative (Figure 2a and 2b). The mean SNP distance between this closest relative and the rainbow trout clade is 165 SNPs. Assuming the same clock rate as above, we estimate that the fish clade diverged from the human isolate around 1995. We identified genes carried on a φSa3 prophage that is known to be associated with human immune evasion in most isolates in our sample of CC45, including those from the farmed rainbow trout (Figure 2d). This suggests that this CC is adapted to the human host, and that its presence in other species is a consequence of recent spillover.

CC45 is, in general, methicillin-sensitive (56) and only 2/68 of the isolates in our sample carry the gene associated with methicillin resistance (*mecA*). Genes associated with resistance to other antibiotics are also rare in this CC. Consistent with the low levels of resistance in this clade the isolates from the farmed trout were found to be methicillin-sensitive and carry no resistance genes except for the *blaZ* gene that confers resistance to benzylpenicillin (Figure 2c). Phenotypic resistance was confirmed by selecting two representative isolates, which both matched the genotypic data (Table S2).

We investigated the potential of CC45 isolates to cause Staphylococcal food poisoning by checking for the presence of staphylococcal enterotoxins. The isolates we sampled from the farmed fish carry nine enterotoxin coding genes, which are common in CC45.
Figure 1. The genetic diversity of *S. aureus* isolated from three different anatomical sites in a single farmed rainbow trout from a London fish farm.

A minimum-spanning tree was constructed using an alignment of the 12 fish isolates, created by mapping to the *S. aureus* reference genome, LGA251. Points represent groups of identical isolates, with point size correlated with the number isolates. Due to the low number of SNPs, each SNP was manually checked to ensure that they were not a consequence of mapping error. The colours represent different sampling fish sites: intestine (blue), gills (orange), and skin (green). Isolates extracted using charcoal swabs are indicated in a darker shade, where the tissue samples are indicated in a lighter shade.
Figure 2: *S. aureus* isolates from a single rainbow trout purchased from a fish farm in London, England fall within the diversity of CC45. A maximum-likelihood phylogeny of 68 isolates of CC45 based on an LGA251 reference-mapped alignment with recombination removed and rooted using an outgroup from CC398 (with branches <70 bootstrap support collapsed). Outer rings describe (a) the host groups isolates were sampled from, (b) country, presence and absence of (c) AMR genes, (d) φSa3 prophage functional genes, and (e) enterotoxin genes.
Absence of *S. aureus* in wild population of brown trout

We sampled fish from four habitat types that are likely to show variation in spillover exposure. These are habitats with exposure to (1) human, (2) livestock, and (3) avian hosts, as well as (4) very isolated sampling sites (Figure 3). We aimed to sample 30 fish from each of our habitats and collect a total number of 120 fish (see methods). While our eventual sample size was 123, we were unable to reach the desired number of trout samples from the sea (exposure to human populations). We sampled 123 brown trout from 16 sites from four habitats (between 1-11 fish from each loch). We collected water and sediment samples from 23 sites from four habitats and three additional sites not categorised into any of the habitats (Tables S3-S5). All researchers carrying out fishing and sampling were swabbed and found to be negative for *S. aureus*.

We did not detect any positive samples in any of the 123 wild brown trout, nor in their environment (Table 1). Overall, these results suggest that *S. aureus* is either absent or present at very low prevalence in Scottish lochs.

### Table 1: Prevalence of *S. aureus* in the environment and from brown trout in different habitats.

<table>
<thead>
<tr>
<th>Habitat</th>
<th>No. of sampled lochs/sites</th>
<th>No. environment samples</th>
<th>No. of positive environmental samples</th>
<th>No. trout samples</th>
<th>No. of positive isolates</th>
<th>Prevalence in trout</th>
</tr>
</thead>
<tbody>
<tr>
<td>All habitats</td>
<td>16</td>
<td>32</td>
<td>0</td>
<td>123</td>
<td>0</td>
<td>0 (0-0.03)</td>
</tr>
<tr>
<td>Bird Lochs</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>0 (0-0.08)</td>
</tr>
<tr>
<td>Isolated Lochs</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>48</td>
<td>0</td>
<td>0 (0-0.07)</td>
</tr>
<tr>
<td>Livestock Lochs</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0 (0.12)</td>
</tr>
<tr>
<td>Human (Sea)</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0 (0.71)</td>
</tr>
</tbody>
</table>
Figure 3. Location of all the sampling sites in Wester Ross, Scotland. The sites include bird, isolated and livestock lochs and sea. The map was constructed using R (53) Leaflet library (v.2.0.4.1).
Discussion

Our study did not detect *S. aureus* in wild populations of brown trout, nor in the Scottish lochs that they inhabit. All sites with different (although all relatively low) levels of exposures to species that are known hosts of *S. aureus*, including livestock, humans and birds, were negative for *S. aureus*. This suggests the absence or a very low prevalence of *S. aureus* in these populations. Our results are consistent with another study testing for the presence of *S. aureus* from different species of 168 wild fish caught by trawling and rod fishing in sea populations from Japan, where no *S. aureus* was detected (57). Overall, these results argue that the presence of *S. aureus* in farmed fish and fish products is not due to the adaptation of *S. aureus* to wild fish populations.

Previous studies have documented the presence of *S. aureus*, including MRSA, in fish farms and in fish products (32,33). We detected *S. aureus* in both internal and external organs of a single farmed fish using aseptic techniques, suggesting that its presence is not due to contamination via handling. *S. aureus* could be adapted to and persisting within the fish host. However, the *S. aureus* genomic diversity within the single fish is consistent with a date of acquisition (i.e., approximately two years) that is likely older than the lifespan of the fish (the fish weight was 300g, which is consistent with an age of less than eight months (58)). Given the lack of clustering by anatomical location, it is more likely that the fish acquired *S. aureus* through passive filtration of its external environment. Previous studies suggested that MRSA can also survive for extended periods of time in the sea and river, and marine fresh water (59,60).

*S. aureus* may be adapted to the conditions within aquaculture. We detected the blaZ gene in the fish farm isolates. B-lactamase production (encoded by blaZ gene) renders *S. aureus* resistant to benzylpenicillin, phenoxyimethylenicillin, ampicillin, amoxicillin, piperacillin and ticarcillin (61). Amoxicillin and ampicillin are commonly used in aquaculture worldwide (62,63). Therefore, the blaZ gene could provide *S. aureus* with a selective advantage over other non-resistant bacteria within this environment. However, our results show that the fish farm rainbow trout isolates are part of CC45, which is associated with both nasal colonization and bloodstream infections in humans (56). The fish farm isolates nest within a more diverse clade of human isolates and contains a φSa3 prophage, which encodes a human immune evasion cluster (64). Our results therefore suggest that the presence of *S. aureus* in this aquaculture environment is due to recent spillover from human populations. This is consistent with another study that showed the presence of a CC398 MSSA isolate from a gilthead seabream (35), which most likely originated from a human-associated lineage of CC398 (22,36,37). *S. aureus* have been recovered from municipal (65–68), hospital (69), and agricultural wastewaters/sewage (70), representing potential sources of human environmental contamination.

We detected nine enterotoxin genes in all our farmed fish isolates. Staphylococcal enterotoxins have also been detected in other studies of *S. aureus* in fish and fishery products (71,72). Staphylococcal food poisoning is caused by the ingestion of any of the 27 characterised Staphylococci enterotoxins (27,28). The toxins can have neurotic or superantigenic activity which result in vomiting or fever respectively (73). The Staphylococci enterotoxins have high tolerance to denaturing conditions, such as low pH or heat and even when ingested in low quantities, can be toxic to humans (74).
This suggests that fish from aquaculture could constitute a risk of food poisoning (73,74).

This study also optimized a sampling approach using farmed fish to calibrate a S. aureus isolation protocol. Previous techniques for S. aureus isolation relied on the fish being euthanized, whereas this approach allowed fish to be returned to their environment after sampling (32,75,76).

The absence of S. aureus in wild fish populations combined with whole genome sequencing from a farmed fish, suggest that the presence of S. aureus in fish is the result of spillover from source populations. Nevertheless, wider whole genome sequencing of S. aureus from aquaculture, including the environment and fish farm workers, is needed to determine the origin and mechanisms of persistence of S. aureus in this environment. Fish and seafood consumption is predicted to increase by 27% by 2030 and much of this increase will be supplied by growth in the aquaculture sector (77). Aquaculture is known to introduce and amplify new pathogens (78), and it relies heavily on antibiotics to combat infectious diseases (30,79,80). While our study found no evidence of adaptation of S. aureus to fish, growth in the size and density of fish farms could create more opportunities for S. aureus to adapt to aquaculture and to fish and could also promote the evolution and spread of strains that are resistant to antibiotics. This would have potential impacts on both food security and human health.

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References


38. Geography | Highland profile – key facts and figures | The Highland Council [Internet]. [cited 2022 Aug 17]. Available from: https://doi.org/10.1101/2022.10.18.512561; this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.


