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Shining Light on Oral Biofilm Fluorescence In Situ Hybridization (FISH): Probing the Accuracy of In Situ Biogeography Studies

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ABSTRACT

The oral biofilm has been instrumental in advancing microbial research and enhancing our understanding of oral health and disease. Recent developments in next-generation sequencing have provided detailed insights into the microbial composition of the oral microbiome, enabling species-level analyses of biofilm interactions. Fluorescence in situ hybridization (FISH) has been especially valuable for studying the spatial organization of these microbes, revealing intricate arrangements such as "corncob" structures that highlight close bacterial interactions. As more genetic sequence data become available, the specificity and accuracy of existing FISH probes used in biogeographical studies require reevaluation. This study examines the performance of commonly used species-specific FISH probes, designed to differentiate oral microbes within in situ oral biofilms, when applied in vitro to an expanded set of bacterial strains. Our findings reveal that the specificity of several FISH probes is compromised, with cross-species hybridization being more common than previously assumed. Notably, we demonstrate that biogeographical associations within in situ oral biofilms, particularly involving *Streptococcus* and *Corynebacterium*, may need to be reassessed to align with the latest metagenomic data.

1 | Introduction

The oral biofilm, being readily accessible for sampling, has provided microbial specimens for scientific exploration since the invention of light microscopy (Leewenhoeck 1684). Consequently, oral microbes have played a significant role in scientific discoveries and research across medical, ecological, and molecular fields (Kreth and Merritt 2023, Merritt and Kreth 2023, Peng et al. 2022, Verma et al. 2018). Over the past two decades, next-generation sequencing has offered comprehensive insights into the oral microbiome, identifying the specific microbial composition within the biofilm and pinpointing individual microbes with species-level resolution (J. L. Baker et al. 2024). This has facilitated many studies on species-specific interactions that influence oral biofilm development. However, the spatial and temporal distribution of oral microbes is influenced not only by adjacent species in the biofilm but also by host and environmental factors (Lamont et al. 2018). Fluorescence in situ hybridization (FISH) has been especially instrumental in depicting a more accurate picture of the in situ oral microbiome, providing a refined picture of their spatial arrangement (Barbosa et al. 2023, Gu et al. 2022). FISH, in general, has uncovered complex microbial biogeographies, allowing for large-scale analyses with high taxonomic resolution (Azimi et al. 2022). Additionally, oral microbiome sequence data enable FISH to incorporate noncultivable species, shining light on how these uncharacterized organisms integrate into the

broader biofilm structure and aid in biofilm development. Thus, FISH-based biogeography of the oral biofilm offers essential, biologically relevant context for microbiome studies. While FISH studies on selected cultivated oral microbes in the in vitro biofilm context have been done since the early 2000s (Thurnheer et al. 2004), the development of several new fluorochromes for labeling FISH probes and their integration into multiplex approaches (such as CLASI-FISH) allowed for a three-dimensional analysis of the in situ microbial arrangement of dental plaque–specific microbes (Valm et al. 2012, Valm et al. 2016, Valm et al. 2011). In their seminal 2016 publication, Jessica Mark Welch and colleagues utilized metagenomic sequencing of supragingival plaque samples from healthy volunteers, combined with CLASI-FISH, to investigate the intricate assemblages and in situ structures of microbial biofilms (Mark Welch et al. 2016). A key outcome of the FISH analyses was the prominent presence of corncob structures, featuring a filamentous bacterial species at the core, surrounded by coccoid cells in a characteristic corncob-like arrangement. These structures and their microbial composition were visualized using specific genus-level FISH probes for *Corynebacterium* (Cor633) and *Streptococcus* (Str405) (Mark Welch et al. 2016). The visual abundance of both species was also observed in the metagenomic sequencing results (Mark Welch et al. 2016). These findings inspired numerous follow-up studies, including investigations from our group (Redanz et al. 2021, Treerat et al. 2023, Treerat et al. 2022, Treerat et al. 2020, Morillo-Lopez et al. 2022, Wilbert et al. 2020). We were particularly interested to investigate the molecular basis for the interspecies interactions occurring between these organisms, prompting us to isolate several oral *Corynebacterium* species to use in coculture studies with oral streptococci. Notably, it was observed that extracellular membrane vesicles produced by *Corynebacterium durum* and *Corynebacterium matruchotii* could induce specific phenotypes in *Streptococcus sanguinis*, reshaping its transcriptomic profile to enhance commensalism (Treerat et al. 2023, Treerat et al. 2020, Kreth et al. 2024).

The study of corncob formations also addressed fundamental questions in microbial ecology, such as habitat specialization. While the aforementioned FISH study primarily examined the biogeographical distribution of *Corynebacterium* and *Streptococcus* at the genus level, more detailed follow-up research defined the taxon specificity of the corncob structure's interacting partners (Morillo-Lopez et al. 2022). The spatial organization in these biofilms suggests that microbes selectively interact with specific partners, thereby fostering community stability. This finding reinforces the concept that defined microhabitats are occupied by a distinct set of microbial species (Morillo-Lopez et al. 2022). Moreover, their study confirmed that biogeographical FISH analyses can provide valuable insights that advance the molecular understanding of oral microbial ecology.

A major challenge when employing FISH on undefined samples, such as in situ oral biofilms, is that FISH probe design relies on available published sequences. When the number of sequences is small, the specificity of designed FISH probes can appear very high, as all available sequences can be aligned to select the best-matching probe. However, as an increasing number of sequences become available, probes that were initially used to define bacterial species, genera, groups, or domains may prove to be less specific than previously assumed, and therefore, they should be regularly validated (Amann and Fuchs 2008). The 16S rRNA gene is a highly conserved region of the bacterial genome that encodes for the small ribosomal subunit, making it essential for protein synthesis. Its conserved and variable regions allow for the identification and differentiation of bacterial species through sequencing and phylogenetic analysis. The issue with 16S rRNA reliability was identified early on with the widely used fluorescent EUB338 probe (Daims et al. 1999), which binds to a conserved region of the 16S rRNA and is commonly used to quantify the eubacterial domain. Following the publication of EUB338 in 1990, which was based in design on 11 16S rRNA sequences (Amann et al. 1990), an increase in available and analyzed 16S rRNA sequences revealed that EUB338's coverage of the eubacterial domain was not as broad as initially thought (Daims et al. 1999). The authors concluded that the coverage of bacterial probes, as with all rRNA-targeted probes, must be regularly reassessed, and modifications or new probe designs should be implemented as needed (Daims et al. 1999).

Given the steady increase in oral microbiome sequencing studies and the resulting availability of more 16S rRNA sequences for individual species, it is crucial to assess how accurately previously defined species-specific FISH probes can discriminate among species. In this study, it was found that biogeographical associations in in situ oral biofilms, particularly for *Streptococcus* and *Corynebacterium*, may need to be revisited to accurately reflect the latest metagenomic information.

2 | Materials and Methods

2.1 | Bacterial Growth and Preparation for FISH

All bacterial species listed in Table 1 were grown in Brain Heart Infusion media (Bacto BHI; Becton Dickinson & Co., MD, USA). *Actinomyces oris* and *Actinomyces viscosus* T14VJ1 were grown in strictly anaerobic conditions (90% N_2, 5% CO_2, 5% H_2; v/v) at 37°C. All other species listed in Table 1 were grown in aerobic conditions with 95% air, 5% CO_2 (v/v) at 37°C. After sufficient growth ($A_{600} > 1$), species were centrifuged for 10 min at 3000 rpm with an A-4-62 Rotor (Eppendorf 5810R centrifuge) to remove media. Cells were resuspended in 50% ethanol for fixation and stored at 4°C overnight. Briefly, 100 µL of each sample was added to PCR tubes and micro-centrifuged for 10 min at 7500 rpm/3200 × *g* to remove ethanol. A total of 100 µL of permeabilization solution (0.5 M Tris-HCl pH 7.2, dH_2O, 0.5 M EDTA pH 8, 35 mg Lysozyme) was added, and samples were incubated for at least 15 min at 37°C. Samples then went through a series of dehydration with 50%, 80%, and 100% ethanol for 3 min each, followed by centrifugation (10 min, 7500 rpm/3200 × *g*; Eppendorf 5425 Centrifuge) to remove ethanol.

2.2 | FISH

Oligonucleotide probes listed in Table 2 were synthesized by Integrated DNA Technologies (IDT, IA, USA) and tested for probe specificity on the species listed in Table 1. Briefly, 1 µL of a 100 ng fluorescent probe was added to 50 µL of hybridization buffer (5 M NaCl, 1 M Tris/HCl pH 7.2, 25% formamide, dH_2O, 0.01% SDS) and was incubated at 46°C for at least 2 h. Formamide

TABLE 1 | Bacterial strains.

Strains	Characteristics	Reference
Corynebacterium durum	Low-passage isolate	This study
Corynebacterium matruchotii ATCC14266		
Streptococcus sanguinis SK36	*S. sanguinis* wild type	Xu et al. 2007
Streptococcus sanguinis SK115	Received from Dr. Todd Kitten	S. P. Baker et al. 2019
Streptococcus sanguinis SK49	Received from Dr. Todd Kitten	S. P. Baker et al. 2019
Actinomyces oris	Low-passage isolate	This study
Actinomyces viscosus T14V-J1	Received from Dr. Hui Wu	Gibbons et al. 1988
Streptococcus mutans UA159	*S. mutans* wild type	Ajdic et al. 2002
Streptococcus gordonii V288	Received from Dr. Mark Herzberg; initially identified as *S. sanguis*	Macrina et al. 1982
Streptococcus parasanguinis	Low-passage isolate	This study
Streptococcus pyogenes NZ131	*S. pyogenes* wild type	McShan et al. 2008
Streptococcus sanguinis VMC66	Received from Dr. Kitten	Baker et al. 2019

concentrations of 15% and 35% were also used to test the effect on hybridization. Following hybridization, samples were micro-centrifuged (10 min, 7500 rpm/3200 × *g*) and then resuspended in wash buffer (5 M NaCl, 1 M Tris/HCl pH 7.2, 0.5 M EDTA, 0.01% SDS) for 15 min at 48°C. Samples were micro-centrifuged again and resuspended in TE buffer. Samples were analyzed microscopically using an Olympus IX73 inverted fluorescent microscope with UPlan FLN 100×/1.30 oil objective lens and excitation wavelengths of 587 and 395 nm. Images were acquired using imaging software platform cellSens (Olympus). Exposure times were determined automatically to acquire similar exposed images and to avoid under- or overexposure.

2.3 | Fluorescence Intensity Measurement

To test the fluorescence intensity of each replicate, the imaging program, ImageJ, was used. Images were converted to grayscale, and the mean gray value of 10 distinct areas was collected by circling the fluorescent cells and using the measure tool. To control for the fluorescent variation, the background area was also measured and subtracted from the fluorescent measurements. To determine fluorescence intensity percentage, the following equation was used:

$$\frac{(\text{Mean gray value (MGV) of fluorescence} - \text{MGV background})}{\text{MGV fluorescence}} \times 100.$$

3 | Results

3.1 | In Silico Re-Evaluation of the *Streptococcus mitis* Group FISH Probe Smit371

Previously published FISH probes have the advantage of being extensively evaluated in vitro to demonstrate a specific distribution of microbial species within the context of a community. A recent publication addressing habitat specialization in dental biofilms identified corncob structures between corynebacteria and streptococci (Morillo-Lopez et al. 2022). The study developed specific probe sets to determine whether all streptococci or only particular *Streptococcus* species associate with corynebacteria. The Smit371 probe was designed to specifically target *Streptoccoccus mitis*, *Streptococcus oralis*, *Streptococcus infantis*, and *Streptococcus cristatus* and therefore could distinguish these species from other oral streptococci, such as the abundant *Streptococcus gordonii* and *S. sanguinis* (Morillo-Lopez et al. 2022). The probe was tested against available ATCC strains of the respective species. It was confirmed that under the specified hybridization conditions, the tested *S. sanguinis* and *S. gordonii* strains did not hybridize with the Smit371 probe (Morillo-Lopez et al. 2022). Here, the Smit371 probe sequence (5′-CAATGGACGGAAGTCTGACC-3′) was used to perform a BLASTn search against all available *Streptococcus* sequences. The standard database, core nt, was employed to search for similar sequences using default algorithm parameters (search date range November/December 2024). The organism selection criteria included (1) *Streptococcus* genus (taxid: 1301); (2) *S. gordonii* (taxid: 1302); and (3) *S. sanguinis* (taxid: 1305). Three different searches were performed to determine if the returned result would differ when a specific genus versus specific species were used. The lineage report for selection (1) returned 127 hits in the *Streptococcus* genus, with most hits corresponding to uncultured *Streptococcus* species (66 hits), *Streptococcus agalactiae* (16 hits), and *Streptococcus pneumoniae* (13 hits). Sequence alignments showed a max score of 40.1, with sequence coverage and identity of 100% (Figure S1). The report for selection (2) also showed 100% sequence coverage and identity for 13 published *S. gordonii* strains (Figure S2). Additionally, 29 of the 100 returned sequences exhibited one mismatch in the middle of the sequence alignment, indicating the potential for hybridization with the Smit371 probe. The BLASTn search (3) for *S. sanguinis* strains demonstrated 100% sequence coverage and identity for 17 out of the 38 returned sequences, with alignment scores of 40.1 (Figure S3). As expected, the *S. sanguinis* ATCC 10556 strain was not among the identified sequences. In summary, BLASTn sequence analysis of Smit371 identified 100% matches with some published *S. sanguinis* and *S. gordonii* sequences. This suggests that the Smit371 probe might not be suitable for distinguishing *S. mitis*, *S. oralis*, *S. infantis*, and *S. cristatus* from *S. sanguinis* and *S. gordonii*.

3.2 | Specificity of Previously Published *S. sanguinis* Probe

A specific probe for *S. sanguinis* has been reported previously and has been shown to successfully hybridize with *S. sanguinis* ATCC10556 (Li et al. 2014). To determine whether this probe (referred to here as Ssan1) could effectively discriminate *S.*

TABLE 2 | FISH probes.

Probe name	Sequence 5′ to 3′	Purpose	Modification	Reference
Ssan1	GCATACTATGGTTAAGCCACAGCC	FISH probe for *S. sanguinis* 16S rRNA	5′ Alexa Fluor 488 (NHS Ester)	Li et al. 2014
Ssan2	CGTGACAAACGACGAAA	FISH probe for *S. sanguinis* SK36 23S rRNA DNA coding sequence	5’ Alexa Fluor 488 (NHS Ester)	This study
Ssan3	TTTCGTCGTTTGTCACG	FISH probe for *S. sanguinis* SK36 23S rRNA gene sequence	5’ Alexa Fluor 488 (NHS Ester)	This study
Cor633	AGTTATGCCCGTATCGCCTG	FISH probe for Corynebacterium 16S rRNA	5’ Alexa Fluor 546 (NHS Ester)	Mark Welch et al. 2016
Eub338	GCTGCCTCCCGTAGGAGT	Universal FISH probe for domain bacteria	5’ Alexa Fluor 488 (NHS Ester)	Amann et al. 1990

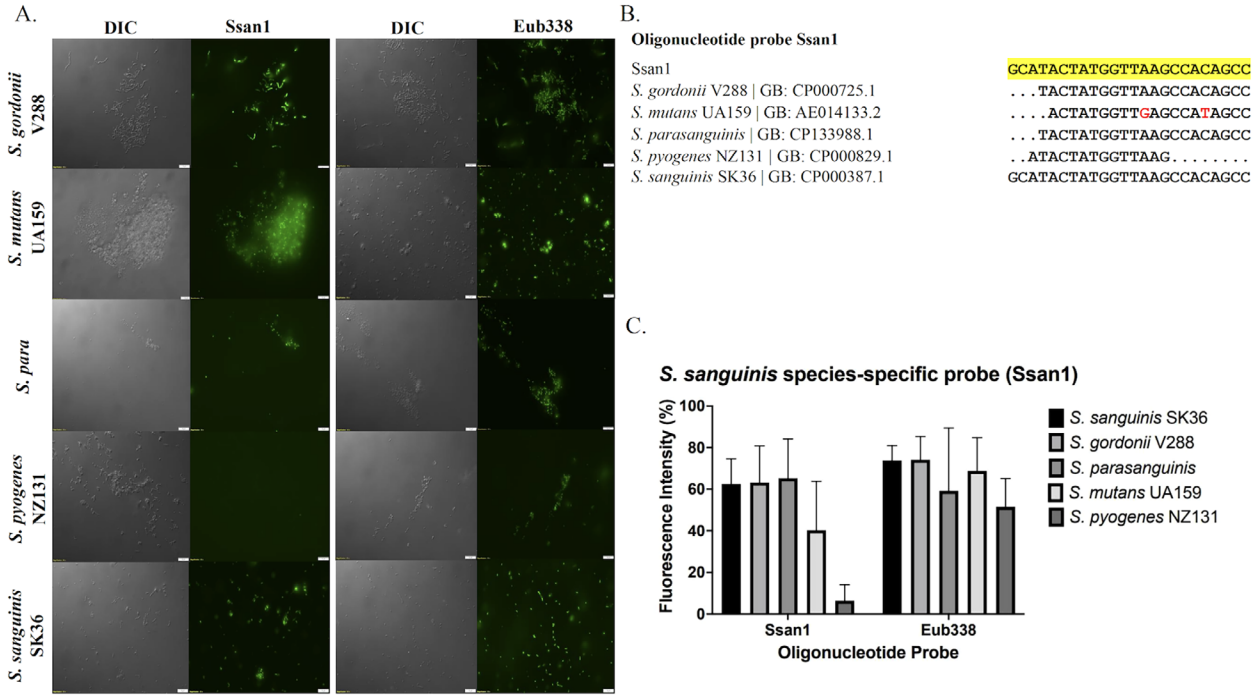

FIGURE 1 | (A) FISH microscopy of *Streptococcus sanguinis* species-specific probe (Ssan1) and Eubacteria probe (Eub338) with indicated strains. (B) Oligonucleotide sequence alignments for strains compatible to *S. sanguinis* species-specific probe Ssan1 using BLASTn against the core nt database. (C) Fluorescence intensity averages of species involved in FISH with probes Ssan1 and Eub338. *S. para.*—*S. parasanguinis*.

sanguinis from other oral streptococci, FISH was performed as described in Section 2. Strains to be tested were chosen from closely related oral streptococcal species, such as *S. gordonii* (grouped with *S. sanguinis* in the Gordonii subclade) and *Streptococcus parasanguinis* (which forms its own subclade but is part of the larger Mitis clade that includes the Gordonii subclade), as well as the more distantly related *Streptococcus mutans* and *Streptococcus pyogenes*, each of which forms its own subclade (Patel and Gupta 2018). A fluorescent signal was observed for *S. gordonii* strain V288, *S. mutans* strain UA159, *S. parasanguinis*, and *S. sanguinis* SK36, while *S. pyogenes* NZ131 did not produce significant fluorescence (Figure 1A). The Eub338 control probe, as expected, demonstrated universal hybridization to all tested species, confirming that the lack of hybridization of Ssan1 to *S. pyogenes* NZ131 was not due to experimental errors.

Quantification of fluorescent signal intensity revealed that the Ssan1 probe produced consistent signal intensities across *S. sanguinis* SK36, *S. gordonii* V288, and *S. parasanguinis*, with slightly lower intensity for *S. mutans* UA159 and very low intensity for *S. pyogenes* NZ131 (Figure 1B). Sequence alignments with the Ssan1 probe (Figure 1C, Ssan1 highlighted in yellow) confirmed a 100% match with *S. sanguinis* SK36, while *S. gordonii* V288 and *S. parasanguinis* showed very close sequence coverage. The slightly reduced fluorescence intensity for *S. mutans* UA159 could be attributed to a two-base mismatch with the Ssan1 probe. The weak to negligible fluorescence intensity observed for *S. pyogenes* NZ131 is well-supported by the lower homology coverage, particularly at the 3′-end, which is critical for stable hybridization between the probe and its target. In conclusion, while the Ssan1 probe demonstrates strong affinity for *S. sanguinis* ATCC10556 (Li et al.

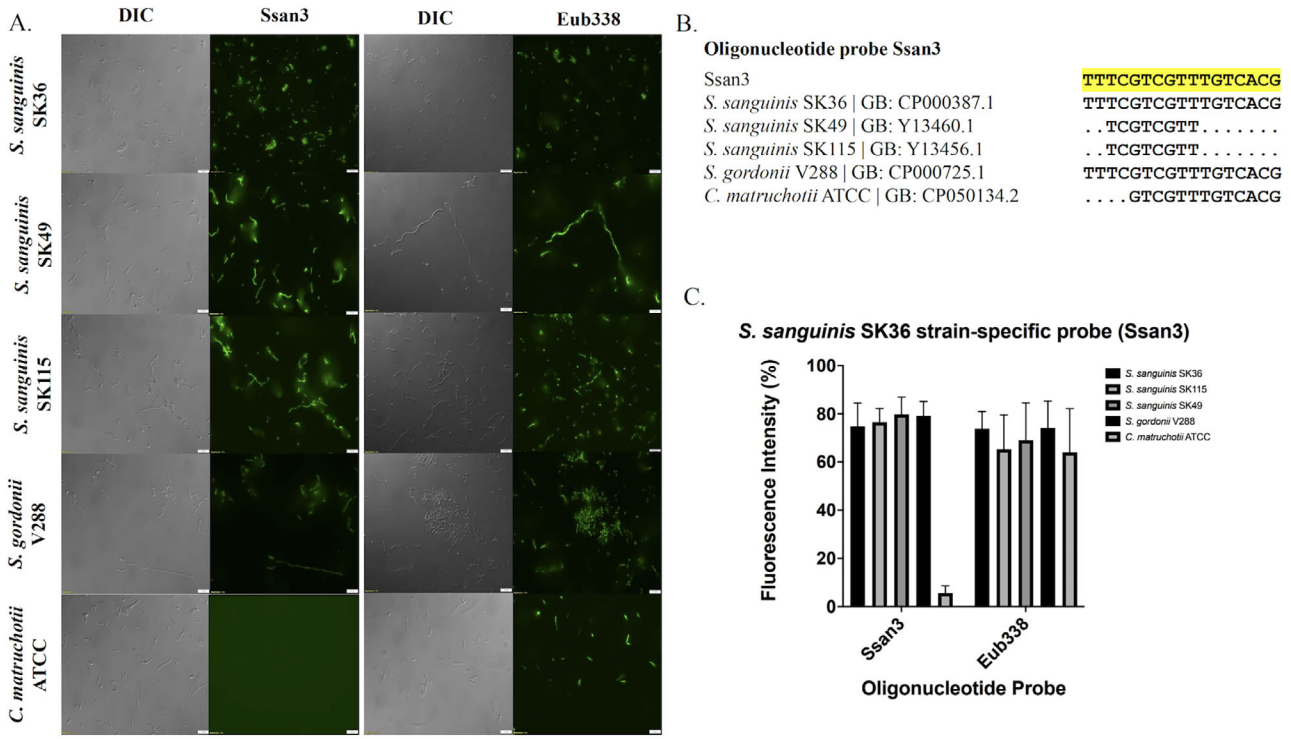

FIGURE 2 | (A) FISH microscopy of *Streptococcus sanguinis* SK36 strain-specific probe (Ssan3) and Eubacteria probe (Eub338) with indicated strains. (B) Oligonucleotide sequence alignments for strains compatible to *S. sanguinis* SK36 strain-specific probe Ssan3 using BLASTn against the core nt database. (C) Fluorescence intensity averages of species involved in FISH with probes Ssan3 and Eub338.

2014) and SK36 (our findings), it does not effectively discriminate against other oral streptococci.

3.3 | *Streptococcus sanguinis* Might Not Be Distinguishable From Other Streptococci by a Single Probe

A new *S. sanguinis*-specific probe, Ssan3, was designed based on the *S. sanguinis* SK36 23S rRNA gene sequence. The selection of the 23S rRNA gene instead of the 16S rRNA was based on previously published research showing that the 23S rRNA gene is suitable for the design of FISH probes and has been suggested as alternative to 16S rRNA (Fuchs et al. 2001). To test the specificity of Ssan3, we used two other *S. sanguinis* strains, *S. gordonii* V288 and *C. matruchotii* (a Gram-positive species found in close proximity to oral streptococci [Morillo-Lopez et al. 2022] but belonging to a different class of bacteria) as a negative (phylogenetically distant) control. As expected, during in vitro FISH, the probe did hybridize with *S. sanguinis* SK36. However, the probe also produced a clear signal with *S. gordonii* V288 and strong signals for *S. sanguinis* SK115 and SK49. No signal was observed for probe Ssan3 with *C. matruchotii* (Figure 2A). Hybridization with the Eub338 control probe resulted in fluorescent signals for all tested strains, confirming the efficacy of our hybridization protocol (Figure 2A). In silico analyses revealed 100% sequence identity of the probe to SK36 and V288, confirming the observed fluorescence signals with both species. Interestingly, while we could not observe a visual difference in fluorescent intensities between the three tested *S. sanguinis* strains, probe Ssan3 seemed to have much lower homology to SK49 and SK115, with only eight out of 17 nucleotides matching (Figure 2B). We observed that lower sequence homology can result in diminished fluorescence of probe in some cases, demonstrated by Ssan1 and *S. pyogenes* NZ131 (Figure 1). The fluorescence images were further supported by fluorescence intensity quantification (Figure 2C). We also designed probe Ssan2 using the SK36 23S rRNA DNA coding sequence; thus, the probe should not hybridize against the 23S rRNA. This probe served as a negative control, and, as expected, no signal was detected against SK36, SK49, and SK115 (Figure S4). Unexpectedly, we did observe fluorescent signals with *S. sanguinis* VMC66 and *S. pyogenes* NZ131. The signals were not uniform among all cells but did lead to fluorescent signaling that seemed to originate from some chains (Figure S4). These results suggest that even when a FISH probe successfully hybridizes with the intended *S. sanguinis* strains, other streptococcal species may also produce positive fluorescence signals. Furthermore, a critical limitation was identified with the negative control probe, which should not hybridize with any target but still produced a distinguishable signal for some cells. This highlights the potential for false positives, a significant concern when analyzing undefined samples.

3.4 | Evaluation of the Corynebacterial FISH Probe Cor633

Oral *Corynebacterium* have garnered significant interest due to their abundance in the oral cavity (Mark Welch et al. 2016). The corynebacterial FISH probe Cor633 has been instrumental in characterizing the biogeographical distribution of corynebacterial species within dental plaque (Mark Welch et al. 2016). Interestingly, while cultivating the corynebacterial species within a selective medium (Tsuzukibashi et al. 2014) previously employed

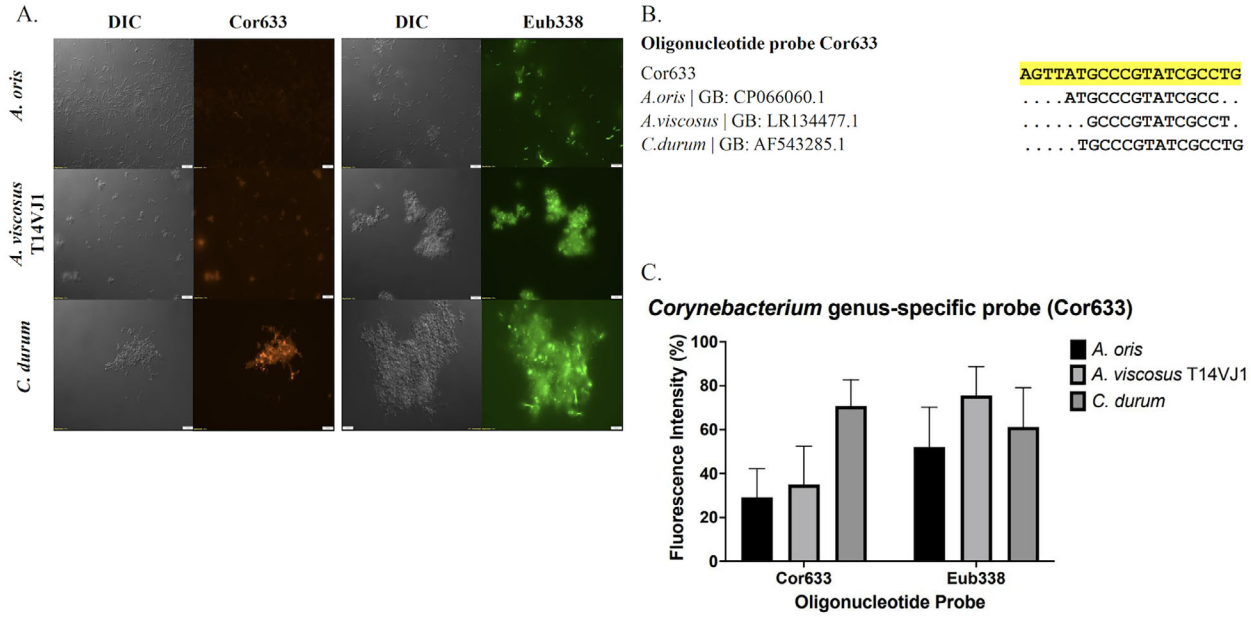

FIGURE 3 | (A) FISH microscopy of *Corynebacterium* genus-specific probe (Cor633) and Eubacteria probe (Eub338) with indicated strains. (B) Oligonucleotide sequence alignments for strains compatible to *Corynebacterium* genus-specific probe using BLASTn against the core nt database. (C) Fluorescence intensity averages of species involved in FISH with Cor633 and Eub338.

for isolating *C. durum* (Treerat et al. 2020), an unexpected result was encountered. Newly isolated strains, which were initially phenotypically identified as resembling *C. durum*, were later determined through chromosomal sequencing to belong to the *Actinomyces* genus (Treerat et al. 2022). This finding prompted an evaluation of the ability of Cor633 to hybridize with *A. oris* and *A. viscosus*. For validation, *C. durum* was used as a positive control, although the probe had previously only been tested with *C. matruchotii*, which is considered more abundant in dental plaque than *C. durum* (Mark Welch et al. 2016, Esberg et al. 2020). As shown in Figure 3A, Cor633 successfully hybridized with *C. durum*, producing bright fluorescent signals. Interestingly, weaker fluorescent signals were also observed for both *A. oris* and *A. viscosus*, still allowing for the identification of individual cells (Figure 3A). Probe Eub338, used as a universal control, produced fluorescent signals for *C. durum*, *A. oris*, and *A. viscosus*. Sequence alignment of Cor633 revealed a 100% match to the *Corynebacterium* genus. However, mismatches were noted in the 16S rRNA sequences of *A. oris*, *A. viscosus*, and *C. durum* (Figure 3B). Quantification of fluorescent signal intensities supported the visual observations. Cor633 produced consistent signals for *C. durum*, albeit with some cross-hybridization to *Actinomyces* species. For Eub338, average fluorescent signals were shown, demonstrating a consistent pattern (Figure 3C).

3.5 | Influence of Formamide on the Stringency of FISH Probes Cor633 and Ssan1

Formamide is widely used with FISH to regulate the stringency of hybridization between probes and their nucleic acid targets. The optimal denaturing conditions for specific hybridization are determined using formamide dissociation profiles, which typically reveal a trend of decreasing brightness in hybridized cells as the formamide concentration increases (Yilmaz and Noguera 2007). To assess the influence of formamide on the performance of the FISH probes Cor633 and Ssan1, we tested hybridization at formamide concentrations of 15%, 25%, and 35% (Figure 4A). Concentrations were chosen based on published results showing a relationship between formamide concentration used and fluorescence intensity (Thurnheer et al. 2004). Results indicated that increasing the formamide concentration within this range had no significant effect on fluorescent intensities (Figure 4B).

4 | Discussion

Recent studies employing FISH to investigate the biogeographical distribution of species within oral biofilms have provided significant insights into the oral microbiome (Valm et al. 2012, Valm et al. 2011, Mark Welch et al. 2016, Morillo-Lopez et al. 2022, Wilbert et al. 2020). However, these studies often overlook challenges related to probe design and experimental methodologies. Thus, it is important to consider careful evaluation of multiple parameter that could influence FISH accuracy, especially for FISH studies on undefined multispecies samples. Some of the parameters are discussed in the following sections.

The process of designing FISH probes typically involves validation using domesticated reference microbial strains (Mark Welch et al. 2016, Morillo-Lopez et al. 2022, Wilbert et al. 2020, Thurnheer et al. 2001). This approach assumes that wild-type strains in situ will hybridize under the same conditions optimized for domesticated strains cultivated in artificial bacterial growth media. However, the ecological and physiological context in situ is markedly different, as microbes in the oral cavity grow in saliva and interact with a diverse microbial community. In general, environmental growth conditions influence transcriptional profiles to a certain extent when compared to bacterial growth medium (Lewin et al. 2022, Aprianto et al. 2018). Cellular properties of microbes are also influenced (Dalhoff 1985), potentially impacting the hybridization efficiency of FISH probes.

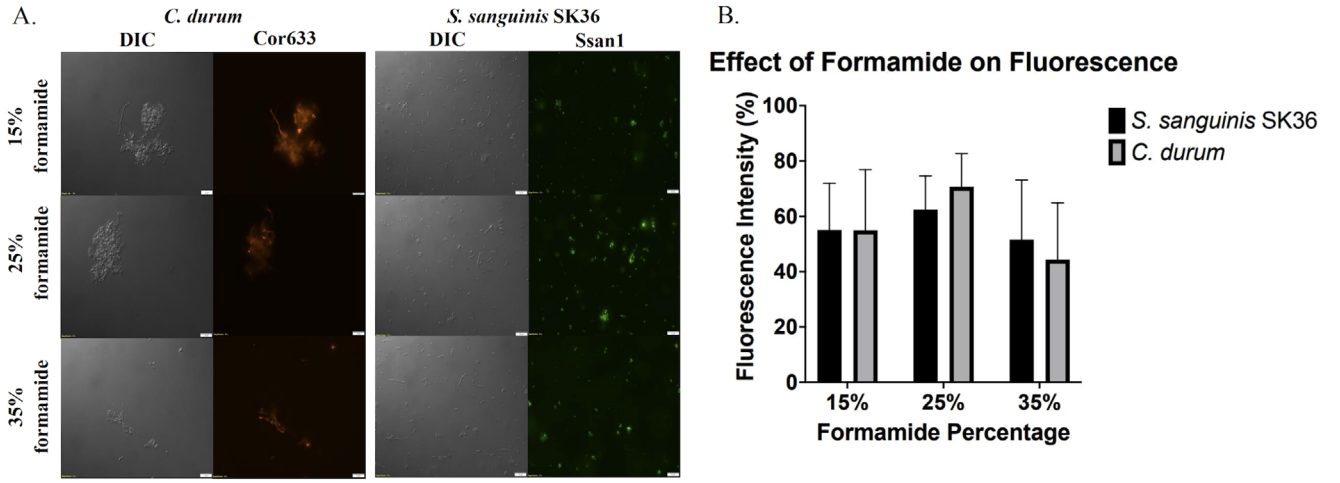

FIGURE 4 | (A) Effect of changing formamide concentration in hybridization buffer on *Corynebacterium durum* and *Streptococcus sanguinis* SK36. Probes Cor633 and Ssan1 were used in hybridization. (B) Fluorescence intensity averages of indicated species with the indicated percent formamide used in the hybridization buffer.

While the use of domesticated strains offers practicality and repeatability, it introduces discrepancies between the behavior of laboratory-adapted and wild-type strains. Prolonged cultivation of microbial strains in artificial media often results in the loss of traits critical for survival in their native environments, a phenomenon widely observed across diverse microorganisms (McLoon et al. 2011, Kennedy et al. 2021, Le Senechal et al. 2021, Steensels et al. 2019). For instance, laboratory strains of *Bacillus subtilis* have been shown to carry a mutation in the *epsC* gene, leading to decreased exopolysaccharide production (McLoon et al. 2011). Since exopolysaccharides influence the local microenvironment and affect probe diffusion, such mutations could alter FISH probe penetration. Additionally, laboratory strains often exhibit reduced biofilm-forming capabilities, a critical survival trait in natural habitats (Leiman et al. 2014, Krol et al. 2019). FISH probes are commonly optimized for planktonic microbes, but their performance in biofilm-associated bacteria, which grow within a complex extracellular matrix, remains less understood. The in situ biofilm matrix can impede probe diffusion and cellular entry. Variability in biofilm phenotypes has been documented for *S. mutans* (Zhou et al. 2018, Palmer et al. 2013), suggesting that strain-specific biofilm characteristics may influence hybridization efficiency. This phenotypic variability extends to other oral microbes and is likely the norm rather than the exception (S. P. Baker et al. 2019, Biyikoglu et al. 2012, Velsko et al. 2018). in situ, microbial cells exhibit diverse physiological states, further complicating FISH analysis. Actual ribosomal numbers, critical for an even probe hybridization among a population, are heterogenous on the population level and vary among cells depending on their growth phase and physiological condition (Brettner and Geiler-Samerotte 2024). Probes optimized for exponentially growing laboratory strains with a homogenous ribosome content may not account for these variations. For example, FISH analysis of defined *Lactobacillus* species revealed that up to 20% of cells failed to stain with species-specific probes, even with an optimized three-enzyme pretreatment for cell permeabilization (Quevedo et al. 2011). Similarly, variability in FISH probe hybridization affinity has been reported for *Corynebacterium* in situ, with some cells frequently remaining unstained (Morillo-Lopez et al. 2022). This phenomenon has been attributed to differences in cell permeability or ribosomal content (Morillo-Lopez et al. 2022). The enzymatic treatment used to permeabilize cells for FISH can also introduce variability. Enzymes must diffuse through the biofilm matrix to reach individual cells, and matrix components may interfere with this process. Prolonged enzymatic treatments have been shown to alter community structure and microbial abundances compared to mechanical disruption (Zhang et al. 2018), thus potentially leading to bias toward easily permeabilized cells. While nonstaining cells may be attributable to permeability issues, the misidentification of species is also a plausible explanation. For instance, isolates phenotypically resembling *Corynebacterium* have been reclassified as *Actinomyces* based upon more rigorous molecular characterizations (Treerat et al. 2022). These findings highlight the importance of considering the limitations of probe design and experimental methodologies in FISH studies of microbial biogeography. Addressing these challenges is critical for accurate characterization of microbial communities in situ.

Given the results of the in vitro experiments performed in this study, it is evident that FISH probe specificity must be held to a high standard. The genus-level, species-level, and strain-level probes failed to specifically bind to only the template genus, species, or strain, a problem that has been reported for group-specific probes in environmental samples as well (Barr et al. 2010). Probe Cor633 hybridized with *Actinomyces* species, probe Ssan1 hybridized with *Streptococcus* species other than *S. sanguinis*, and probe Ssan3 hybridized with *S. sanguinis* SK115 and SK49, all of which showed poor alignments according to NCBI BLAST (see Figure 2B). To further investigate this apparent specificity issue, we performed BLAST searches using FISH probes employed in Morillo-Lopez et al. (2022) to image the oral microbiome. This study suggests that probes Smit651 and Scri995 may hybridize nonspecifically with *S. sanguinis* strains as well as other species not identified as *S. cristatus*, *Streptococcus sinensis*, or members of the *S. mitis* group (see Table 3). There is potential that using these probes to map an oral biofilm spuriously labels cells as *S. cristatus*, *S. sinensis*, or a member of the *S. mitis* group, leaving doubts about the accuracy of corresponding FISH results. Furthermore, these concerns over probe reassessment cast doubt on the dependability of FISH results over time. If the specificity of probes degrades as

TABLE 3 | Sequence alignments of FISH probes Scri995 and Smit651.

Probe	Strain or species	Sequence	Sequence ID
Scri995	*S. cristatus*, *S. sinensis*	TAGGACGGGCACCGGGAT	
	S. sanguinis NCTC10904	TAGGACGGGCACC….	LR134002.1
	S. sanguinis SK36	TAGGACGGGCA…….	CP000387.1
	S. periodonticum KCOM 2412	TAGGACGGGCACCGGGAT	CP034543.1
	S. sanguinis CGMH010	…….GGCACCGGAT	CP040556.1
	S. pneumoniae 20.2T	TAGGACGGGCACCGGGAT	MT807605.1
Smit651	*S. mitis* group	CCCCTCTTGCACTCAA	
	S. sanguinis SK36	CCCCTCTTGCA….	CP000387.1
	S. sanguinis K52	CCCCTCTTGCACTCAA	MF578809.1
	S. sanguinis IMAU11196	CCCCTCTTGCACTCAA	KT887244.1
	S. sanguinis 1	CCCCTCTTGCACTCAA	MK027245.1
	S. sanguinis ATCC 29667	CCCCTCTTGCACTCAA	AY281085.1

more microbial sequences become available, FISH probe design may need to be revisited and reworked regularly. If retesting probes is necessary for continued accuracy and reliability, is FISH an ideal method for species identification of unknown samples?

For domesticated strains, often the medium used for proliferation to optimize FISH probe hybridization conditions represents classic laboratory conditions; yet, in the context of oral biofilm hybridization, it is important to consider a medium that reflects the environment in situ. Artificial saliva medium (ASM) or saliva-supplemented medium could offer bacteria more natural conditions, enhancing relationships with neighboring bacteria, representing a host environment, and supporting important ecological phenomena that scientists are discovering today (Daims et al. 1999). These phenomena may not occur in the less desirable laboratory conditions often used for FISH, as the bacteria lack critical environmental influences found in situ. Additionally, FISH success is limited to the experimental design, which varies upon the species. Within the FISH procedure, there are vital steps for success, which include fixation, permeabilization, and probe hybridization (Moter and Gobel 2000). Fixation reagents differ for Gram-negative and Gram-positive bacteria, with varying concentrations of ethanol used for Gram-positive and formaldehyde for Gram-negative bacteria (Thurnheer et al. 2001, Lukumbuzya et al. 2019). Permeabilization allows the probe to enter into the cell for hybridization and can be more challenging for recent oral isolates that retain wild-type traits, which could challenge permeabilization of the cell membrane, necessitating a longer permeabilization period for oral isolates. Formamide concentrations used in hybridization are contingent on the thickness of the peptidoglycan layer found in the cell (Batani et al. 2019). All these specificities in reagents make FISH a limiting procedure in the context of a multispecies environment, reducing the value of the experimental outcome.

Live-and-dead staining was another instance of a critical assay having been reassessed for its credibility. In principle, the assay differentiates live cells from dead cells based upon a dye's ability or inability to penetrate through the cell membrane. Netuschil et al. (2014) state that there are limitations with this protocol, reaffirmed by the BacLight provided protocol, that the dye mixture must be predetermined and specific to the bacteria in question due to differences in the nucleic acid content affinities from one dye to another. An analogous obstacle is present within FISH, which necessitates prospective users to consider the specific context of experiments, as it is not a universal procedure and there are many parameters to adjust to achieve accurate, unambiguous results.

Taking in consideration the experimental revisions outlined here, successfully utilizing FISH for biogeographical studies of the oral microbiome requires great attention to detail and commitment to accuracy. For further studies, we suggest that probe design considers the context of the in situ biofilm matrix, referencing recent oral isolates for specificity tests, if possible. Additionally, we suggest that the experimental design be reconsidered, with alternate growth mediums used to reflect the natural oral environment, as well as close attention paid to the fixation, permeabilization, and hybridization steps to ensure that proper reagent concentrations are used for the bacterial species in question. These revisions are especially important in the context of clinical diagnostics, for example, using FISH for pathogen detection (Claesson et al. 2022), as inaccurately identifying pathogens could lead to improper treatment plans for patients.

In conclusion, FISH is a powerful technique that has provided significant insights into the arrangement of microbial species in the oral cavity, yielding information crucial to the current understanding of microbial ecology. These studies are invaluable, as they can guide the development of novel approaches to understanding interspecies interactions. However, they come with an important caveat: potential promiscuity among widely used FISH probes. While 16S rRNA is a convenient target, it may not always provide the precision required, as discussed elsewhere (Johnson et al. 2019, Usyk et al. 2023). To enhance the reliability of FISH, probe design may need to incorporate other conserved genes for hybridization. As the saying goes, "a picture is worth a thousand words," but it is incumbent upon the user to select the most accurate tools to capture that picture.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data are available upon reasonable request.

Peer Review

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.