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# Fucoidans from *Laminaria hyperborea* demonstrate bactericidal activity against diverse bacteria

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## Abstract

Fucoidans are a heterogenous class of fucose-rich sulfated carbohydrates which have attracted increasing attention in cancer and inflammation research due to their bioactive properties. There are reports that fucoidans may have direct antibacterial effects and synergy with antibiotics. However, the literature is conflicting, potentially due to the limited reporting of origin, characteristics, and extraction methods of the fucoidans tested. Here we report the results of 18 defined fucoidans screened for direct, indirect, and synergistic antibacterial effects. 15 distinct fucoidan fractions, isolated from *Laminaria hyperborea* using a solvent-free extraction process, were characterised for molecular weight, pH, viscosity, and sulfur content. These, together with three commercially available crude fractions, were assessed at concentrations from 0.03125–24 mg mL<sup>-1</sup> for minimum inhibitory concentration against *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus sanguinis*. Furthermore, we tested a selection of fucoidans for antibacterial synergy with vancomycin and indirect antibacterial effects in whole blood survival assays. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed to assess the stress response in fucoidan-treated *S. aureus* cultures. We have identified one fucoidan fraction with bactericidal activity against diverse bacteria. This effect is dose-, fucoidan fraction- and bacteria-specific, and furthermore, not related to osmotic stress. No synergistic effects were observed with fucoidan in combination vancomycin. Fucoidans have exciting potential as antimicrobial agents. Further analysis is required to establish the precise molecular characteristics responsible for their potent bactericidal activity.

**Keywords** Bioactive carbohydrates · Sulfated carbohydrate · Fucoidans · Antibacterial · *Staphylococcus aureus*

## Introduction

Fucoidans are a heterogenous class of fucose-rich sulfated carbohydrates found in various species of brown marine algae, echinoderms and seagrasses. They have been widely used in dietary supplements, functional foods, cosmetics

and as aquaculture feed supplements. However, there is growing interest in their potential therapeutic applications. Accordingly, the biological effects of fucoidans have been the focus of much research, particularly in the fields of inflammation and cancer. A number of comprehensive reviews have been published in these areas (Luthuli et al. 2019; Wang et al. 2019; Apostolova et al. 2020). Briefly, fucoidans have been shown to have diverse activity

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including scavenger receptor modulation, immune activation, anti-angiogenesis, the blockade of metastasis, mobilisation of stem cells and interference with SDF1/CXCR4 axis, anti-oxidant and pro-oxidant effects and selectin blocking (Baba et al. 1988; Itoh et al. 1993; Irhimeh et al. 2007; Wang et al. 2010; Park et al. 2011; Park et al. 2013; Kim et al. 2014).

This study focuses on the potential antibacterial effects of fucoidan. Emerging evidence suggests that fucoidan may exhibit antibacterial effects against a range of bacterial pathogens (Lee et al. 2013; Liu et al. 2017; Jun et al. 2018; Ayrapetyan et al. 2021). Fucoidan's potential antibacterial activity is thought to be multifaceted, involving various mechanisms such as inhibition of bacterial cell wall synthesis, interference with bacterial adhesion and biofilm formation (Jun et al. 2018; Liu et al. 2019), and modulation of the host immune response to enhance bacterial clearance.

At a cellular level, fucoidan has been shown to delay apoptosis in neutrophils and induce the production of pro-inflammatory cytokines interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF)- $\alpha$  (Jin and Yu 2015). In addition, Fucoidan induces the production of TNF- $\alpha$  and IL-1 in macrophages (Hsu et al. 2001) as well as upregulation of cytotoxicity in natural killer (NK) cells *in vitro* (Hsu et al. 2001). *In vivo*, fucoidan has been shown to increase the secretion of IL-12 and promote the maturation of bone-marrow derived dendritic cells (Kim and Joo 2008). The same authors demonstrated subsequent increased activation of antigen specific cluster of differentiation (CD)4 and CD8 positive T cells. Studies have reported fucoidan's capacity to disrupt bacterial biofilms, protective matrices formed by bacteria that often confer resistance to antibiotics (Jun et al. 2018). Fucoidan's ability to inhibit biofilm formation and disperse existing biofilms could potentially enhance the susceptibility of bacteria to antimicrobial agents.

Fucoidan has demonstrated promising *in vivo* antibacterial activity against clinically relevant bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus mutans* (Lee et al. 2013; Jun et al. 2018; Ayrapetyan et al. 2021). Depolymerized fucoidans extracted from *Saccharina japonica* show a dose-dependent antibacterial effect against both *E. coli* and *S. aureus* (Liu et al. 2017; Sun et al. 2023). Notably, potency varied relative to the bacterial strain and specific fucoidan. Structural factors within fucoidan molecules have been suggested as key determinants of their antibacterial activity. Low molecular weight (<500 kDa), abundant sulfate content, and uronic acid content emerged as potential factors contributing to their effectiveness (Liu et al. 2019; Luthuli et al. 2019). Similarly, fucoidans isolated from *Fucus vesiculosus* demonstrated a bacteriostatic effect on a range of bacterial strains, including of *E. coli* and *S. aureus*. Microscopic observations showed preserved bacterial cell integrity, but

changes in size and surface roughness, particularly in Gram-positive bacteria (Ayrapetyan et al. 2021).

In addition, there are reports of a synergistic effect in combination with antibiotics. The addition of fucoidan to ampicillin or gentamycin reduced time-kill against a range of common oral bacteria (Lee et al. 2013). This effect is suggested to occur through inhibition of bacterial cell wall synthesis and is supported by similar studies for *Staphylococcus epidermidis*, *P. aeruginosa*, *Enterococcus faecalis*, *S. aureus*, and *E. coli* (Chmit et al. 2014).

One of the key challenges in fucoidan research is their heterogeneity, both in terms of their species of origin, geographical location and the processes used for extraction (Li et al. 2008). Indeed, the biological effects from broadly similar products can be diverse and sometimes contradictory (Kim et al. 2014; Jeong et al. 2017; Miyazaki et al. 2019). Furthermore, there has often been a paucity of specific information regarding the precise molecular weights, extraction methods and origins, in the published literature. As research into this exciting area progresses, a greater emphasis on reporting the specific characteristics of the fucoidan being tested should assist in distinguishing the biological activity associated with distinct fucoidan types, molecular weights, and isolated fractions.

The objectives of this paper are to present the results of 15 distinct fucoidans of known origin and molecular weight as well as three commercially available extractions. We have tested for antibacterial activity against *S. aureus*, *S. mutans* and *Streptococcus sanguinis*. The 18 fucoidans were tested for direct antibacterial activity and a selection were tested for an adjuvant effect with vancomycin. Furthermore, we tested for an indirect antibacterial effect via an immunostimulatory effect on whole blood.

## Methods

**Bacterial strains** *Staphylococcus aureus* (ATCC 25923) was purchased from the German microorganism culture collection [Deutsche Sammlung von Mikroorganismen (DSM)] as DSM strain number 1104. The organism is a methicillin-sensitive *S. aureus* (MSSA) and a human clinical isolate (Moriarty et al. 2017). *Streptococcus mutans* (ATCC 25175) and *Streptococcus sanguinis* (ATCC 10556) were purchased from LGC Nordic (Teddington Middlesex, U K). The freeze-dried bacterial pellets were initially dissolved in 0.5 mL brain heart infusion (BHI) medium with 1% yeast extract, before transfer into 5 mL of extra medium and incubated for 48 h. Stock solutions were then stored at -80 °C until use.

**Fucoidan** The majority of fucoidans (15) were supplied by International Flavours and Fragrances incorporated (IFF) N&H Germany GmbH & Co. KG, Walsrode, Germany. The

fucoïdians were isolated from *Laminaria hyperborea*, using a patented solvent free extraction process (Hjelland et al 2013). Briefly, the seaweed was harvested near the coast of Norway and stored in containers allowing fucoïdan to leach out in water. The exudate solution was collected and purified via ultrafiltration with a molecular weight cut off greater than 10 kDa. Fucoïdan was thus contained in the retentate and isolated as a brownish solid via spray-drying. The remaining three fucoïdians were purchased from Sigma Aldrich (Merck Life Science A/S, Denmark) and isolated from *Fucus vesiculosus* (F8190), *Undaria pinnatifida* (F8315), and *Macrocystis pyrifera* (F8065), respectively.

The MW was determined using size exclusion chromatography. Viscosity was determined by preparing a 1 wt.-% aqueous solution of fucoïdan by dissolving 1.00 g fucoïdan powder in 99.00 g deionized water. The temperature of the solution was equilibrated at 20 °C. The spindle of a Brookfield viscosimeter (LVT) was immersed in the solution and rotated at 60 rpm. After one minute, the viscosity was read off at the viscosimeter. pH was measured using the same 1 wt.-% aqueous solution equilibrated at 20 °C. The electrode of a pH meter was immersed in the solution and the pH was read as soon as the indicated value was stable for one minute.

Moisture content was assessed by weighing one gram of the sample to the nearest 0.0001 g in a bottle and heated to  $105 \pm 2$  °C until a constant weight was reached. Then, the weighing bottle was removed from the oven, covered with a lid immediately, cooled in a desiccator for 1 h and weighed to the nearest 0.0001 g. The moisture content was derived from the difference of the two obtained values. The sulfur content was determined according to the Schöniger flask test (MacDonald 1961).

**Minimal inhibitory concentration/minimum bactericidal concentrations assay** Stock solutions ( $48 \text{ mg mL}^{-1}$ ) were made for each type of fucoïdan. Luria-Bertani (LB) medium was used for *S. aureus* and BHI supplemented with 1% yeast extract for *S. mutans* and *S. sanguinis*. 200  $\mu\text{L}$  of stock solution was transferred to each well of the first row of a 96 well microtiter plate (Brand, pureGrade S, Avantor, Denmark) in duplicates. 100  $\mu\text{L}$  of medium was added to each of the other wells and a 2-fold dilution row was made, using 100  $\mu\text{L}$  from the first row. The final row was the positive control (with bacteria, without fucoïdan) and the negative control (without bacteria and fucoïdan). Subsequently, a bacterial suspension in 0.9% NaCl was adjusted to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.1 (equals approximately  $=1.5 \times 10^8$  colony forming units (CFU)  $\text{mL}^{-1}$ ) and diluted in media to reach approximately  $10^6$  CFU  $\text{mL}^{-1}$ . 100  $\mu\text{L}$  of bacterial suspension was added to each well (final concentration:  $5 \times 10^5$  CFU  $\text{mL}^{-1}$ ). Thus, fucoïdan concentrations from 24 - 0.375  $\text{mg mL}^{-1}$  were tested. For each fucoïdan type, a 2-fold dilution

row was made, and 100  $\mu\text{L}$  of sterile media was added instead of the bacterial suspension. This was done to make sure that the fucoïdan was not contaminated and to subtract the background signal from the  $\text{OD}_{600}$  measurements.

A 10-fold dilution row ( $10^{-1}$  –  $10^{-6}$ ) of the bacterial suspension was made and cultured on agar plates (LB agar for *S. aureus* and Chromogenic agar (CHROMID CPS ELITE, Biomerieux-nordic, Denmark) for *S. mutans* and *S. sanguinis*) in duplicates (20  $\mu\text{L}$  dilution $^{-1}$ ) to determine the accurate inoculum. The plates were incubated in a humidified incubator with 5%  $\text{CO}_2$  at 37°C for 18–24 h. After the incubation period, the  $\text{OD}_{600}$  was measured using a SPECTROstar Nano (BMG Labtech) and the minimum inhibitory concentration (MIC) value was determined, after subtracting the background signal from the fucoïdan dilutions.

If inhibition was detected by the OD measurements, cultures from each well were 10-fold serial diluted and cultured on agar plates to determine the CFU  $\text{mL}^{-1}$  at each fucoïdan concentration.

**Minimal inhibitory concentration with vancomycin and fucoïdan** Vancomycin stock solution ( $20 \mu\text{g mL}^{-1}$ ) was made by mixing vancomycin with media. 200  $\mu\text{L}$  ( $20 \mu\text{g mL}^{-1}$ ) vancomycin was added to the first row of a 96 well plate (Brand, pureGrade S, Avantor, Denmark). 100  $\mu\text{L}$  of medium was added to each of the other wells and a 2-fold dilution row was made. A 48  $\text{mg mL}^{-1}$  stock solution of each type of fucoïdan was made in the appropriate medium. 100  $\mu\text{L}$  fucoïdan solution was transferred to each well of column 1 and 7 and a 2-fold dilution row was made from column 1–6 and 7–12. A positive control (with bacteria, without fucoïdan or vancomycin), negative control (without bacteria or fucoïdan) and fucoïdan control (fucoïdan dilution row) was included in each assay. A bacterial suspension was made in 0.9% NaCl with an  $\text{OD}_{600} = 0.1$  ( $\text{OD}_{600} 0.1 = 1.5 \times 10^8$  CFU  $\text{mL}^{-1}$ ). 66.66  $\mu\text{L}$  of the bacterial suspension was transferred to 9.93 mL of BHI broth ( $10^6$  CFU  $\text{mL}^{-1}$ ) and 100  $\mu\text{L}$  of bacterial suspension added to each well (final concentration:  $5 \times 10^5$  CFU  $\text{mL}^{-1}$ ). MIC was determined as above. No CFU measurements were made in the synergy experiments.

**Whole-blood survival assay** A whole blood survival assay was established based on a previously described method (Grønnemose et al. 2017). Whole blood was collected from one healthy male donor in hirudin tubes. An overnight culture of *S. aureus* was suspended in 0.9% NaCl and  $\text{OD}_{600}$  adjusted to 0.1. Fucoïdan was diluted to 100  $\text{mg mL}^{-1}$  in 0.9% NaCl and diluted  $\times 50$  and  $\times 100$  with the blood to reach concentrations of 1  $\text{mg mL}^{-1}$  and 2  $\text{mg mL}^{-1}$  and a final volume of 1500  $\mu\text{L}$ . For the controls without fucoïdan, 0.9% NaCl was added, to reach the same blood dilution. The blood was divided into triplicates in individual tubes (500  $\mu\text{L}$  in each tube) and 5  $\mu\text{L}$  of bacterial suspension added to each

tube. The tubes were incubated for two hours, in an end-over-end mixer. To release potential intracellular bacteria engulfed by neutrophils, to each tube, 500  $\mu\text{L}$  of 0.1% Triton-X were added followed by vortexing and sonication for 5 min. A 10-fold dilution row was made from each tube and 20  $\mu\text{L}$  from each dilution was spotted on LB agar plates and incubated overnight. The CFUs were counted and adjusted to CFU  $\text{mL}^{-1}$  for comparison.

**RNA extraction** Extraction of RNA from *S. aureus* (ATCC 25923) cultures was carried out by a phenol-chloroform extraction. Total RNA was extracted from *S. aureus* cultures terminated in 20 % ice cold ethanol and immediately spun down at  $3214 \times g$  for 10 min at 4 °C. Cell pellets were resuspended in an RNA lysis buffer (4 M guanidinium thiocyanate (GITC), 0.1 mM Tris-HCl (pH 7.5), 10 mM NaAcetate (pH 4.5), 25 mM EDTA, 0.1 % Triton X-100, 2 mM DTT) and disrupted by bead beating with a Bead Ruptor Elite (OMNI International) at  $6.50 \text{ m s}^{-1}$  for 45 s using 0.1 mm Zirconium beads (BeadBug prefilled tubes, 2.0 mL, Sigma Aldrich). The tubes were subsequently centrifuged at  $10,000 \times g$  for 5 min at 4 °C, and the supernatant was transferred to tubes with 700  $\mu\text{L}$  acidic phenol (pH 4.5) and 300  $\mu\text{L}$  chloroform. Tubes were then inverted and heated at 80°C for 4 min, followed by cooling on ice. Subsequently, tubes were centrifuged at  $10,000 \times g$  for 5 min, and the aqueous phase was transferred to 96 % ethanol with Na-acetate (37.5 mM) and precipitated ON. RNA was pelleted by centrifugation ( $20,000 \times g$  for 45 min) and washed in ice-cold ethanol. RNA pellets were resuspended in RNase-free  $\text{H}_2\text{O}$  and stored at  $-80^\circ\text{C}$ .

**Reverse-transcription quantitative PCR** Reverse-transcription quantitative PCR (RT-qPCR) was performed to quantify gene expression in fucoidan-treated *S. aureus* cultures. LB medium with or without sub-MICs of fucoidan (3 mg  $\text{mL}^{-1}$  IFF12 or 12 mg  $\text{mL}^{-1}$  IFF15) was inoculated with  $5 \times 10^5$  CFU  $\text{mL}^{-1}$  and incubated for 24 h at 37°C. 1  $\mu\text{g}$  of RNA extracted from these cultures was treated with 0.2 units RNase-free DNase I (New England BioLabs) in 1x DNase

I Reaction Buffer before cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher). Real-time qPCR of cDNA samples was carried out with RealQ Plus 2x Master Mix Green without ROX (Ampliqon) using supplier recommended PCR settings on a CFX Opus 384 Real-Time PCR System (Bio-Rad). Primers used for real-time qPCR are listed in Table 1.

**Statistics** Potential differences in CFU in the blood survival assay and gene expression in the PCR assays were assessed by one-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism 9.4.0. *p*-values below 0.05 were considered significant.

## Results

**Fucoidan characteristics** The results of the fucoidan characterisation are shown in Table 2. The molecular weights ranged from 69 – 1100 kDa.

**Pilot assays** Initially we tested fucoidans at concentrations up to 2 mg  $\text{mL}^{-1}$ , compatible with systemic therapy. We found no antibacterial or bacteriostatic effect in minimal inhibitory concentration assays. In addition, the minimal inhibitory concentration with vancomycin did not demonstrate any antibacterial synergy between fucoidan and vancomycin (supplementary data). Furthermore, the whole blood survival assay showed no statistical difference in the number of viable bacteria after 2 h incubation with either low (IFF14) or high molecular weight (IFF9) fucoidan compared with controls (supplementary data). Accordingly, we tested all fucoidans at higher concentrations of up to 24 mg  $\text{mL}^{-1}$  in MIC assays.

**Minimal inhibitory concentration/minimum bactericidal concentrations assay** The 18 distinct fucoidans were tested for inhibitory activity against three species of bacteria (*S. aureus*, *S. sanguinis*, and *S. mutans*) at concentrations

**Table 1** DNA oligos used in this study

Name	Sequence (5' -> 3')	Notes
<b>opuD_1_F</b>	AGCTTATACTGAAGCCCTACGT	Forward primer for <i>opuD_1</i> amplification in qPCR
<b>opuD_1_R</b>	ACGTCAATAAAAATCCCAATAGGACC	Reverse primer for <i>opuD_1</i> amplification in qPCR
<b>betA_F</b>	CTTTGATGCAGGTGTTGAAGC	Forward primer for <i>betA</i> amplification in qPCR
<b>betA_R</b>	AACAAAGGCACGTGTTTCAAC	Reverse primer for <i>betA</i> amplification in qPCR
<b>vraR_F</b>	CCAAAGCCCATGAGTTGAAGC	Forward primer for <i>vraR</i> amplification in qPCR
<b>vraR_R</b>	CCTGCATCTAATGCACGATATAC	Reverse primer for <i>vraR</i> amplification in qPCR
<b>gyrB_F</b>	GTTGTAAACGCATTGTCACAAGAC	Forward primer for <i>gyrB</i> amplification in qPCR
<b>gyrB_R</b>	CAGTTGTCTCTGTGAAGATTTCTCC	Reverse primer for <i>gyrB</i> amplification in qPCR

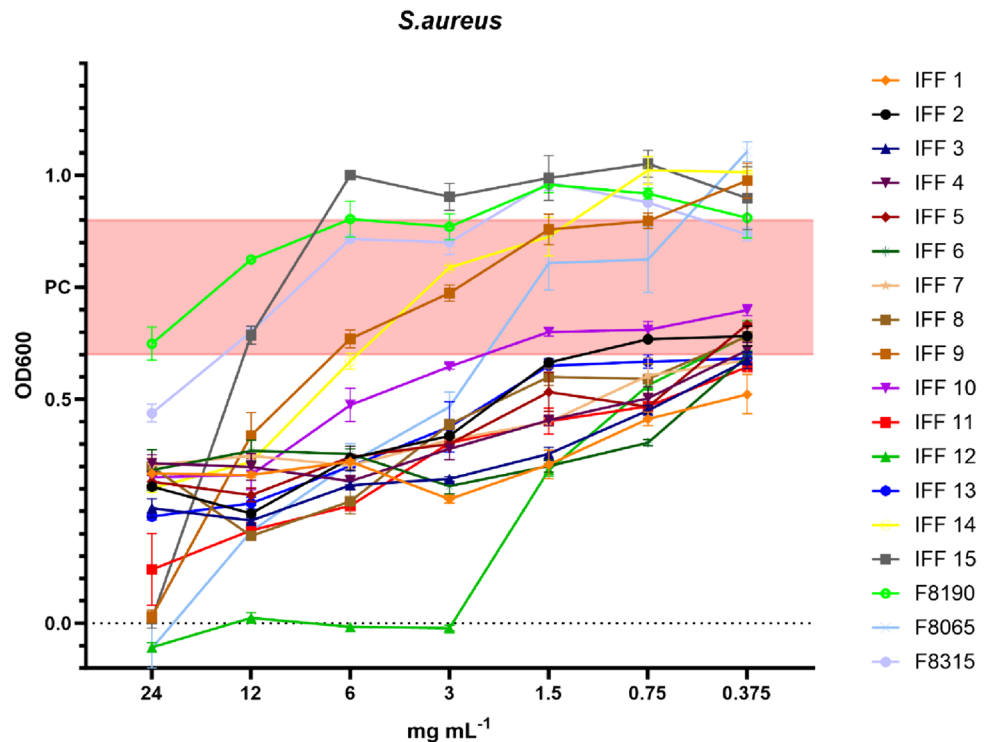
**Table 2** Fucoidan characteristics, including viscosity, pH, moisture, sulfur content, Weight average molecular weight (MW), Number average molecular weight (Mn), Kilodaltons (kDa), and polydispersity (PDI)

Sample name	Mw (kDa)	Mn (kDa)	PDI	Viscosity 1% (mPa s)	pH 1%	Moisture (%)	Sulfur content (%)
<b>IFF 1</b>	460	140	3.29	13.6	7.9	7.4	10.4
<b>IFF 2</b>	1100	600	1.83	5.5	6.0	5.0	11.2
<b>IFF 3</b>	640	230	2.78	5.1	6.0	5.5	12.4
<b>IFF 4</b>	430	120	3.58	3.0	4.7	6.2	11.9
<b>IFF 5</b>	480	150	3.20	7.0	6.1	6.0	12.0
<b>IFF 6</b>	620	240	2.58	9.9	6.8	5.3	11.0
<b>IFF 7</b>	530	190	2.79	6.2	6.4	4.5	8.7
<b>IFF 8</b>	490	160	3.06	7.4	6.6	4.9	8.8
<b>IFF 9</b>	940	380	2.47	6.2	6.2	5.1	11.5
<b>IFF 10</b>	940	270	3.48	10.0	6.2	5.8	10.2
<b>IFF 11</b>	710	340	2.09	7.1	7.0	5.4	10.7
<b>IFF 12</b>	130	30	4.33	5.5	5.9	5.2	8.2
<b>IFF 13</b>	840	350	2.40	5.4	5.9	5.5	8.7
<b>IFF 14</b>	980	130	7.54	8.2	6.5	5.0	12.1
<b>IFF 15</b>	230	150	1.53	3.0	8.9	6.2	37.9
<b>F8190</b>	69	-	-	-	-	-	27
<b>F8135</b>	134	-	-	-	-	-	31
<b>F8065</b>	72	-	-	-	-	-	24

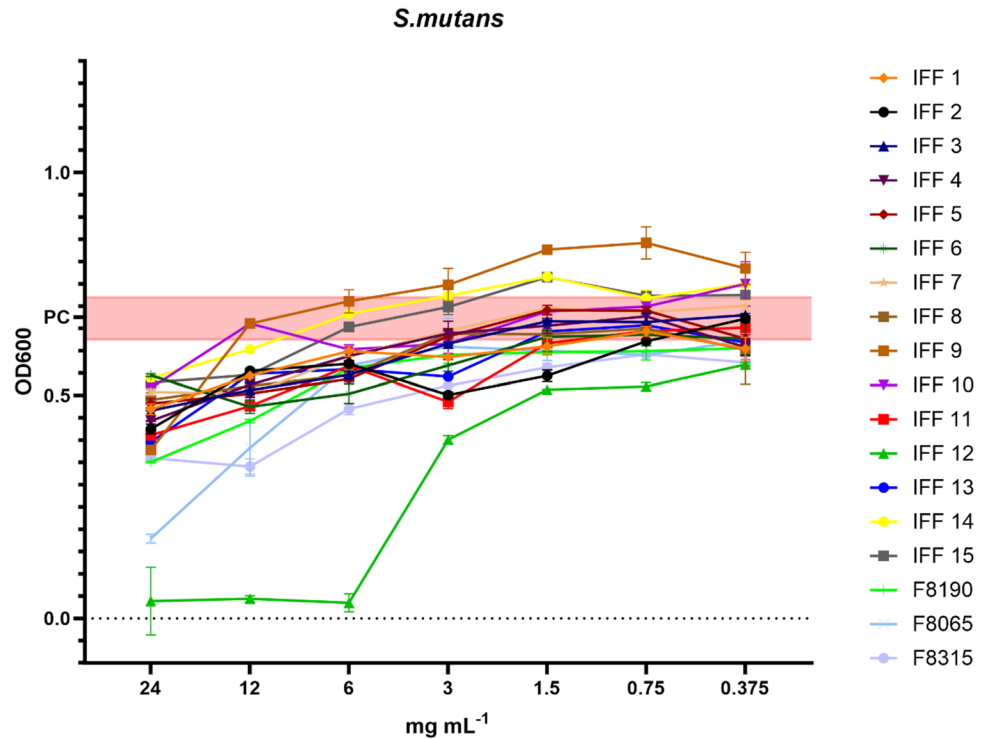
of up to 24 mg mL<sup>-1</sup> (Figs. 1, 2 and 3). Four of the 18 fucoidans demonstrated inhibitory activity. IFF12 showed a bactericidal effect against *S. aureus* and *S. sanguinis* at concentrations of 3mg mL<sup>-1</sup>, and against *S. mutans* at 6 mg mL<sup>-1</sup>. IFF9, IFF15 and F8065 showed an inhibitory

effect against *S. aureus* at 24 mg mL<sup>-1</sup> but not *S. sanguinis* or *S. mutans*. The minimal inhibitory concentration with vancomycin did not demonstrate any antibacterial synergy between fucoidan and vancomycin at any concentration (results not shown). The results of the OD measurements

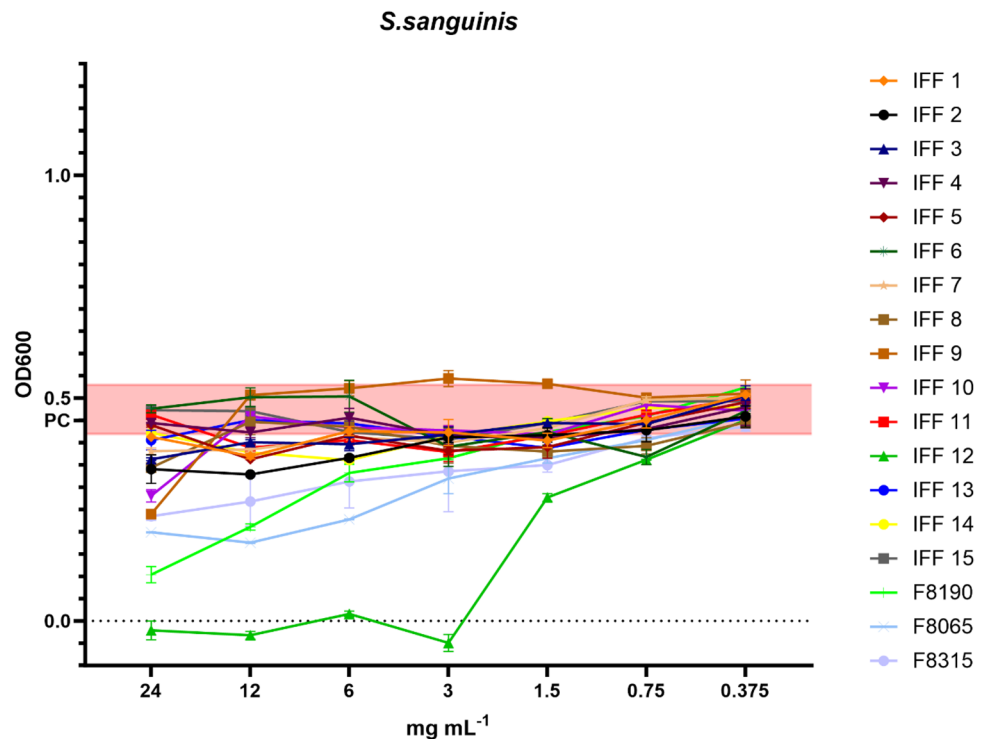
**Fig. 1** Screening of 18 different fucoidan types against *Staphylococcus aureus*. OD<sub>600</sub> values were measured after 18–24 h of treatment. OD values of the positive control (PC) were between 0.6–0.9. Experiments were performed in duplicates (n=2) for each fucoidan type and distributed over three experiments. Data show mean OD<sub>600</sub> values ± standard deviation (SD)



**Fig. 2** Screening of 18 different fucoidan types against *Streptococcus mutans*. OD<sub>600</sub> values were measured after 18–24 h of treatment. OD values of the positive control (PC) were between 0.62–0.72. Experiments were performed in duplicates (n=2) for each fucoidan type and distributed over three experiments. Data show mean OD<sub>600</sub> values ± SD



**Fig. 3** Screening of 18 different fucoidan types against *Streptococcus sanguinis*. OD<sub>600</sub> values were measured after 18–24 h of treatment. OD values of the positive control (PC) were between 0.42–0.53. Experiments were performed in duplicates (n=2) for each fucoidan type and distributed over three experiments. Data show mean OD<sub>600</sub> values ± SD



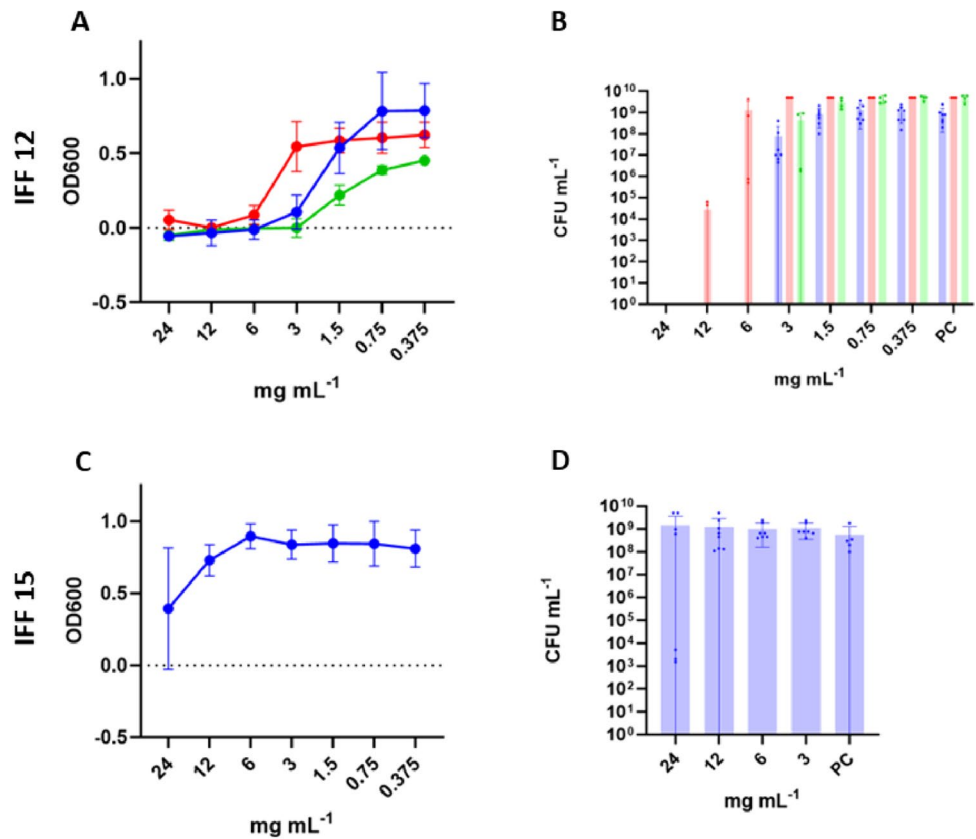
were confirmed by CFU measurements for fucoidans demonstrating inhibitory effects (Fig. 4).

#### Evaluation of osmotic stress induction from fucoidan exposure

The antimicrobial activity of fucoidan IFF12 and IFF15

was observed at high doses (>3 mg mL<sup>-1</sup> or >12 mg mL<sup>-1</sup>, respectively), which could suggest an effect caused by exposure to hyperosmotic conditions, and not a specific antimicrobial effect. To elucidate this, expression of osmo-responsive genes was analysed in *S. aureus* by reverse transcription quantitative

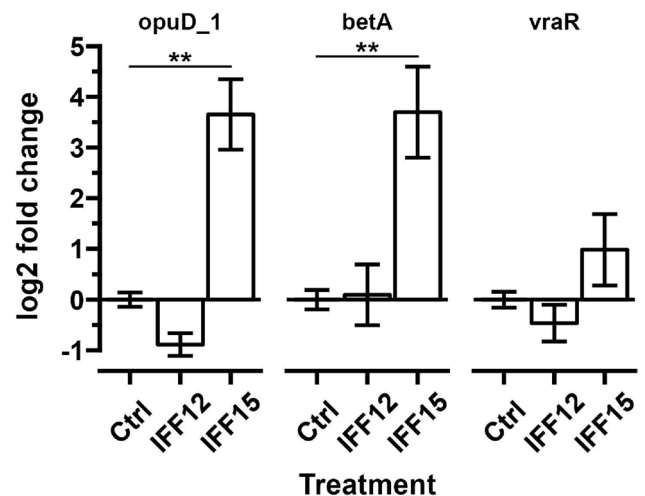
**Fig. 4** Inhibitory/bactericidal effect of two different fucoidan types after 18–24 hours of treatment. IFF 12 was tested against *S. aureus* (blue, seven replicates, three individual experiments), *S. mutans* (red, four replicates, two individual experiments) and *S. sanguinis* (green, four replicates, two individual experiments). IFF 15 was tested against *S. aureus* (blue, 8 replicates, three individual experiments). **A+C.** OD<sub>600</sub> values were subtracted the background from the corresponding fucoidan dilutions without bacteria. Mean±SD. **B+D.** CFU mL<sup>-1</sup>. CFU mL<sup>-1</sup> >5 × 10<sup>9</sup> are shown as 5 × 10<sup>9</sup> CFU mL<sup>-1</sup>. Mean±SD. PC: positive control without fucoidan



PCR (RT-qPCR). This included the oxygen-dependent choline dehydrogenase (*betA*) and the glycine betaine transporter (*opuD\_1*). To explore whether the antimicrobial action involved disruption of cell wall integrity, gene expression of the cell wall stress response gene *vraR* was included in the analysis. *S. aureus* cultures were treated with sub-minimum inhibitory concentrations (sub-MICs) of IFF12 (3 mg mL<sup>-1</sup>) and IFF15 (12 mg mL<sup>-1</sup>) for 24 h, followed by RNA extraction and RT-qPCR. The RT-qPCR analysis revealed a strong significant induction of the osmo-responsive genes *opuD\_1* and *betA* in response to IFF15 (log<sub>2</sub> FC >3), but not to IFF12 (Fig. 5). Significant induction of *vraR* was not observed for either IFF12 or IFF15, indicating no induction of the cell wall stress response. Slightly higher mean values of *vraR* expression was observed upon IFF15 exposure although this increase was not significant. Interestingly, no indications of the activation of the osmotic stress response or the cell wall stress response was observed for IFF12 at sub-MICs, suggesting that the antimicrobial effect of this fucoidan is caused by a different mechanism.

## Discussion

This study focuses on the potential antibacterial effects of 15 different fucoidans extracted using a solvent free extraction process and three commercial preparations



**Fig. 5** Expression of osmo-responsive genes in fucoidan-treated *Staphylococcus aureus*. RT-qPCR was performed on RNA isolated from cultures treated with sub-minimum inhibitory concentrations of fucoidan (3 mg mL<sup>-1</sup> IFF 12 or 12 mg mL<sup>-1</sup> IFF15) for 24 h, and an untreated control culture (Ctrl). Bar charts represent means of log<sub>2</sub> fold change expression levels of *opuD\_1*, *betA* and *vraR* relative to the untreated cultures. Absolute expression levels were normalized to housekeeping gene *gyrB*. Error bars represent standard deviations and asterisk depicts level of statistical significance. (One-way ANOVA with Dunnett's multiple comparisons test. \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ )



against *S. aureus*, *S. mutans*, and *S. sanguinis*. The sulfur content, polydispersity (PDI) and number average molecular weight corresponded broadly to values previously reported for fucoidan (Jun et al. 2018). Importantly we have identified one fucoidan molecule (IFF12) showing significant bactericidal activity against all three species. Furthermore, we have shown that the bactericidal effect in our assays is dose-dependent, and the effect of IFF12 is unlikely to be explained by osmotic stress.

These results are broadly in line with similar studies in this area assessing fucoidan extractions from other sources (Liu et al. 2017; Ayrapetyan et al. 2021). The molecular weights correspond to the lower end of fucoidan fractions described in the literature (Wang et al. 2019). Importantly, as others have identified, these antibacterial effects are highly molecule specific and not a general property of fucose-rich sulfated carbohydrates. Indeed, our results suggest that distinct fucoidan preparations have markedly divergent effects on bacteria. The mechanisms for this are unclear, however, various groups have found a tendency towards greater bioactivity in the lower molecular weight preparations and those with strong anionic properties (Liu et al. 2017). Low molecular weight fucoidans may simply have greater accessibility to biological targets due to their smaller size, enabling more efficient interactions with relevant receptors or bacterial targets. The degree of sulfation may also prove critical in bioactivity, affecting interactions with proteins and enzymes (Wang et al. 2019; Cui et al. 2022). Although we did not assess uronic acid content, other groups have found higher percentages in the lower molecular weight fucoidan fractions (Liu et al. 2017). Uronic acids introduce negative charges to the polymer affecting their overall polyanionic nature, potentially influencing their biological interactions, especially with positively charged molecules (Jones et al. 2004). Interestingly, the fucoidan demonstrating the greatest bioactivity (IFF12), had both the lowest molecular weight and lowest sulfur content of the 15 non-commercially sourced fucoidans tested. Ultimately, further molecular analysis is needed to delineate the particular molecular characteristics and mechanisms contributing to antibacterial potency in our studies. Importantly, the structural characteristics of fucoidan, including its molecular weight, monomer composition, sulfate group content, and their distribution, vary according to extraction methods and the geographical origin and season of harvest of the source algae (Catarino et al. 2018; Ayrapetyan et al. 2021).

As effects were only observed with high doses of fucoidan we speculated that the mechanism of action may simply be related to non-specific effects associated with osmotic stress. The data from analysis of the expression of genes involved with the osmotic stress response of *S. aureus* (*betA* and *opuD\_1*) (Ming et al. 2019; Casey and

Sleator 2021), suggests that the potency of IFF12 is most likely not explained by such a response. Furthermore, there was no observed induction of the cell wall stress response, as evidenced by the absence of significant changes in the expression of the gene encoding the cell wall stress-associated response regulator *VraR* (Belcheva and Golemi-Kotra 2008; Schuster et al. 2020). In contrast, IFF15 which demonstrated antibacterial effects at 24 mg mL<sup>-1</sup> showed evidence of osmotic stress in the exposed bacteria. The antibacterial effects were observed for IFF12 and IFF15 at molar concentrations of 0.023 mmol mL<sup>-1</sup> and 0.052 mmol mL<sup>-1</sup>, respectively. Accordingly, the antibacterial effects observed for IFF15, may be related to osmotic stress, as despite the lower molecular weight of IFF12, and thus relative higher osmolality, higher concentrations of IFF15 were required to achieve antibacterial activity.

Our findings contradict some results for fucoidans previously published. We initially assessed fucoidans at concentrations of up to 2 mg mL<sup>-1</sup>, potentially compatible with systemic therapy, and in line with other studies showing antibacterial activity (Lee et al. 2013). However, we were unable to measure any antibacterial effect at these concentrations. In the study by Lee et al. (2013) the fucoidan used was obtained from Sigma, but without further specification. It is unclear if the products offered by Sigma previously differed in species, purity, or extraction process, which could potentially account for the contradictory results. Furthermore, one cannot exclude that the antibacterial effects were related to contaminants in what is thought to be a relatively crude extract.

The addition of fucoidan to whole blood *ex vivo* did not appear to stimulate bacterial killing by cellular or humoral immune-effectors. In contrast, although not statistically significant, there appeared to be a protective effect. In our assay, we primarily assess the direct bactericidal effects of neutrophils and humoral immune mediators present in whole blood. Neutrophils mediate bacterial destruction via pattern recognition receptors, release of antibacterial peptides and defensins as well as phagolysosomal killing (Flannagan et al. 2009; Grønnemose et al. 2017; Park et al. 2017). However, the documented effects of fucoidan relate mainly to stimulation of cytokine production which, although not directly bactericidal, potentially augment the antibacterial response (Jin and Yu 2015; Miyazaki et al. 2019). As a result, fucoidan may have indirect antibacterial effects including cell recruitment, T-cell differentiation, and macrophage activation, which may not be captured in the incubation period of our study. In particular, recruitment or recruitment augmentation of antibacterial immune cells would not be captured by our *ex vivo* assay. Furthermore, in the final stages of our assays, the cellular components are lysed in order to capture all viable bacteria, including living intracellular bacteria. Consequently,

any phagocytosed bacteria not effectively destroyed within the time frame of our assay would be preserved; thus, potentially obscuring any potential effect occurring at later time points through more complete bacterial opsonisation and engulfment.

**Strengths and weaknesses** We report the results of 15 well-defined and purified fucoidans and 3 commercially available crude fucoidan extracts, tested for direct and indirect antibacterial effects using robust and validated assays. We have identified fucoidans with antibacterial activity. These effects are dose-, fraction- and bacteria-specific, however, further molecular analysis is needed to delineate the precise biological mechanisms contributing to bioactivity.

We have not confirmed previous findings of a synergistic effect with vancomycin over the range of concentrations that we have tested. Furthermore, we do not find increased bacterial destruction in whole blood *ex vivo*. However, due to the *in vitro* design, we cannot conclude that fucoidan may act to support the antibacterial immune response *in vivo*. In addition, we cannot exclude the possibility, that the fucoidans tested may have direct antibacterial effects on other bacterial species or at different concentrations.

**Conclusion** This research contributes to a more comprehensive understanding of the biological effects of fucoidans, a group of marine-derived sulfated polysaccharides. Our data revealed the fucoidans with greatest bactericidal activity, had the lowest sulfur content and molecular weight of the 15 non-commercially sourced fucoidans tested. Interestingly antibacterial activity was not identified in the three commercially sourced fucoidans, despite their comparatively low sulfur content and molecular weights. While the concentrations required for the observed antibacterial effects may limit their application as systemic therapies, there is potential for topical applications or integration into antibacterial coatings. Further investigations in pre-clinical animal models and clinical studies are required to unveil the full extent of fucoidans' antibacterial activity, their potential synergistic effects with antibiotics, and their broader immunomodulatory functions. As research into fucoidans develops, and our understanding of these exciting molecules improves, we hope it will pave the way for developing novel antibacterial therapies.

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1007/s10811-024-03258-2>.

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**Authors' contributions** **Michael L. C. Beagan:** Conceptualization (lead); Project administration (lead); Funding acquisition (equal);

methodology (equal); investigation (equal); writing – original draft (lead); formal analysis (equal); writing – review and editing (lead). **Line L Bang:** Conceptualization (equal); methodology (equal); Investigation (lead); formal analysis (equal); writing – review and editing (equal). **Jens S. Pettersen:** Conceptualization (equal); methodology (equal); Investigation (equal); formal analysis (equal); writing – review and editing (equal). **Rasmus B. Grønnemose:** Conceptualization (equal); methodology (equal); formal analysis (equal); writing – review and editing (equal). **Sebastian Foertsch,** Conceptualization (supporting); resources (supporting); Investigation (equal); writing – review and editing (equal). **Thomas E. Andersen,** supervision (equal); Conceptualization (equal); methodology (lead); formal analysis (equal); writing – review and editing (equal). **Ming Ding.** Funding acquisition (lead); resources (lead); supervision (lead); conceptualization (supporting); writing – review and editing (equal).

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**Data availability** Data are available by request from the corresponding author.

## Declarations

**Competing interests** None

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