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# Synthetic Oligomers Mimicking Capsular Polysaccharide Diheteroglycan are Potential Vaccine Candidates against Encapsulated *Enterococcal* Infections

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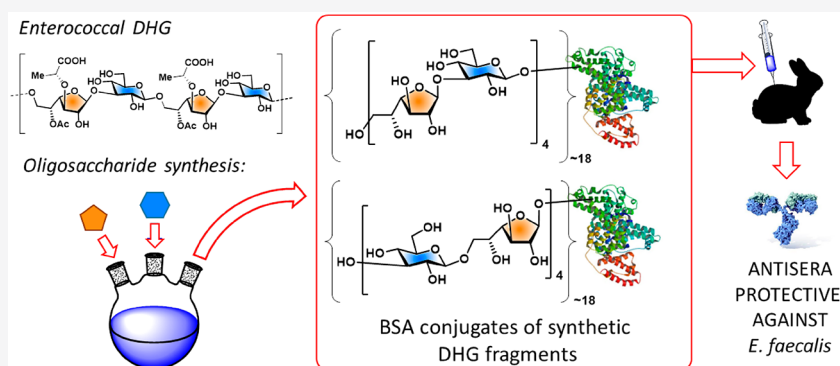
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**ABSTRACT:** Infections caused by *Enterococcus* spp. are a major concern in the clinical setting. In *Enterococcus faecalis*, the capsular polysaccharide diheteroglycan (DHG), composed of  $\beta$ -D-galactofuranose-(1  $\rightarrow$  3)- $\beta$ -D-glucopyranose repeats, has been described as an important virulence factor and as a potential vaccine candidate against encapsulated strains. Synthetic structures emulating immunogenic polysaccharides present many advantages over native polysaccharides for vaccine development. In this work, we described the synthesis of a library of DHG oligomers, differing in length and order of the monosaccharide constituents. Using suitably protected thioglycoside building blocks, oligosaccharides up to 8-mer in length built up from either Galf-Glcp or Glcp-Galf dimers were generated, and we evaluated their immunoreactivity with antibodies raised against DHG. After the screening, we selected two octasaccharides, having either a galactofuranose or glucopyranose terminus, which were conjugated to a carrier protein for the production of polyclonal antibodies. The resulting antibodies were specific toward the synthetic structures and mediated *in vitro* opsonophagocytic killing of different encapsulated *E. faecalis* strains. The evaluated oligosaccharides are the first synthetic structures described to elicit antibodies that target encapsulated *E. faecalis* strains and are, therefore, promising candidates for the development of a well-defined enterococcal glycoconjugate vaccine.

**KEYWORDS:** vaccine, capsular polysaccharide, diheteroglycan, synthetic carbohydrate, *Enterococcus faecalis*, opsonophagocytic assay

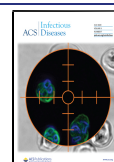
**E**nterococci are one of the most common pathogens associated with nosocomial infections worldwide.<sup>1</sup> Their resistance to multiple antibiotics, including vancomycin and even new generation antibiotics like linezolid, tigecycline, and daptomycin, highlights the urgent need to develop alternative treatments to fight this remarkable opportunistic pathogen.<sup>2,3</sup> Bacterial polysaccharides, such as teichoic acids, capsular polysaccharides, and lipopolysaccharides are considered the most attractive targets for vaccine development, since they represent the bacterial first line of defense against complement and bacterial phagocytes.<sup>4,5</sup> For instance, in enterococci, cell-wall-associated polysaccharides and glycan structures implicated in colonization of epithelial surfaces, inflammation processes, and evasion of host immune system have been

described as promising vaccine candidates.<sup>6–10</sup> Production of a polysaccharide-based vaccine for treatment and prevention of enterococcal infections is a realistic goal, since only a limited number of serotypes seem to exist.<sup>11</sup>

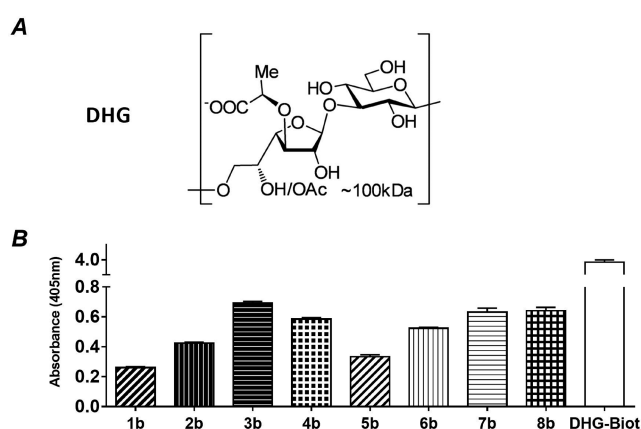
In *Enterococcus faecalis*, four different serotypes (CPS-A–CPS-D) have been described on the basis of analyses of

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different immunoreactivity of antisera raised against different prototype strains by ELISA and opsonophagocytic assay and by genetic analysis of the capsular polysaccharide *cps* locus.<sup>10,12–14</sup> Diheteroglycan (DHG) is a capsular polysaccharide produced by the *cps* locus in *Enterococcus faecalis*, is present in serotypes CPS-C and CPS-D strains, and has been demonstrated to mask lipoteichoic acid (LTA) in the bacteria preventing opsonization by anti-LTA antibodies.<sup>10</sup> The structure of this polysaccharide was first described in 1971 by Pazur et al. as a trisaccharide composed of glucose and galactose backbone with lactosyl and cellobiosyl substituents.<sup>15</sup> However, a more comprehensive structural elucidation of the DHG from *E. faecalis* Type 2 strain by Theilacker and co-workers showed that the polysaccharide repeating unit was  $\rightarrow 6$ )- $\beta$ -Galp-(1  $\rightarrow$  3)- $\beta$ -D-Glcp-(1 $\rightarrow$  with *O*-acetylation in position 5 and lactic acid substitution at position 3 of the Galp residue (see Figure 1A).<sup>10</sup> Finally, the definitive structure of the DHG was established when the R configuration of the lactic acid substituent in the DHG backbone was elucidated by Krylov et al.<sup>16</sup>



**Figure 1.** Structures of the synthetic DHG saccharides and their immunoreactivity with anti-DHG. (A) Structures of the repeating unit of the DHG from *E. faecalis* Type 2. (B) Antibodies raised against native DHG from *E. faecalis* Type 2 were evaluated for their ability to bind specifically the different synthetic DHG resembles of native DHG. Anti-DHG serum was used at final concentration of 41.2  $\mu$ g IgG/mL. Synthetic DHG saccharides, dimers **1b** and **5b**, tetramers **2b** and **6b**, hexamers **3b** and **7b**, and octamers **4b** and **8b** were used to coat the streptavidin coated plate with 0.1  $\mu$ g per well. Biotinylated DHG (DHG-Biot) was used as the control to assess the preferred ability of the anti-DHG serum for the antigenic structures tested. Absorbance was measured at 405 nm after 40 min of incubation with the substrate. Bars represent the mean of data, and the error bars represent the standard error of the mean.

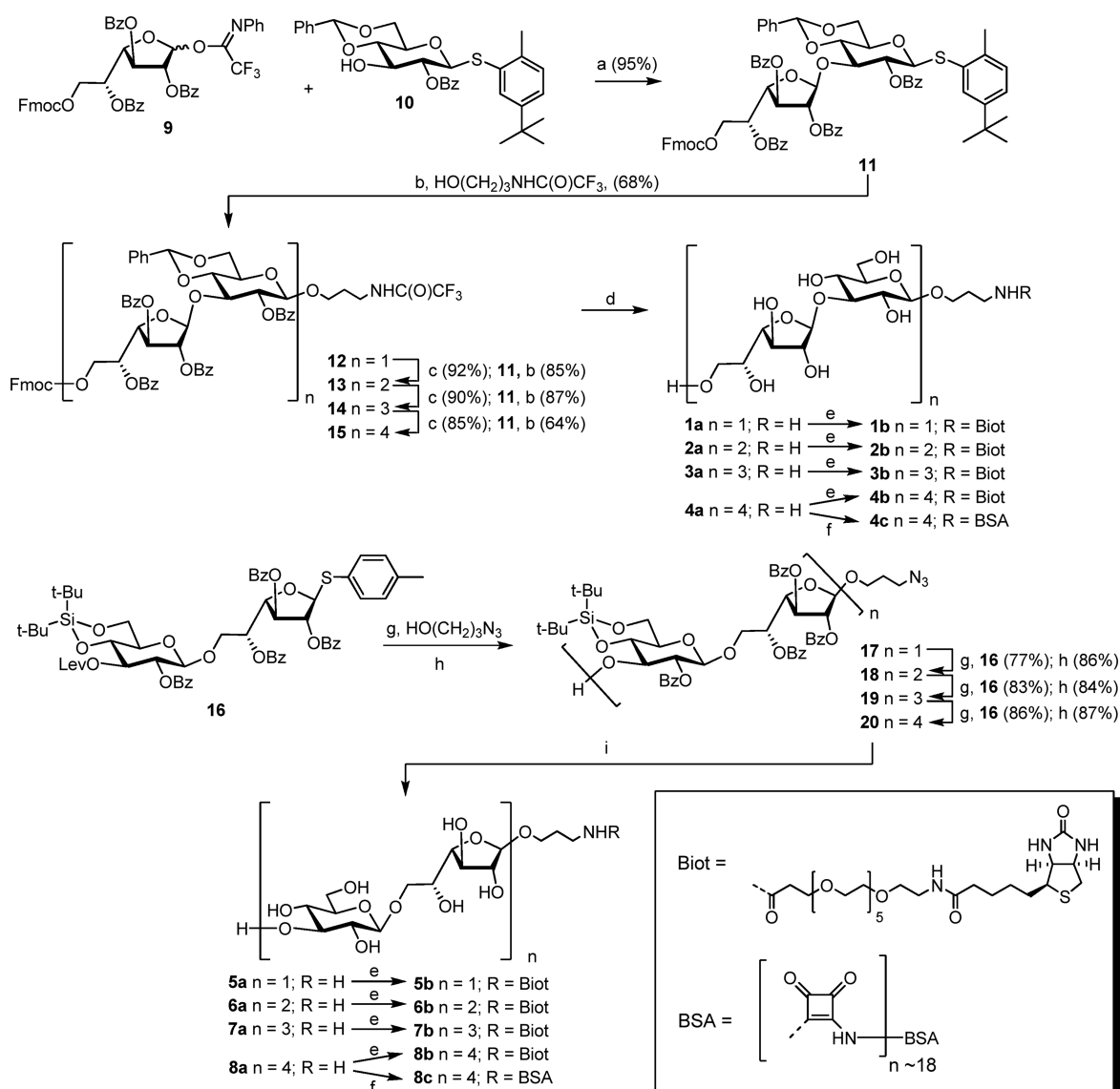
Rabbit antiserum raised against DHG showed good protective efficacy in mice infected with *E. faecalis* strains of CPS-C and CPS-D serotype, making it a promising target for vaccine development to fight infections caused by encapsulated *E. faecalis* strains.<sup>10</sup> However, the development of a polysaccharide-based vaccine is challenging because of the disadvantages associated with antigen purity.<sup>17</sup> Naturally occurring polysaccharides, such as DHG, are microheterogeneous and their purification leads to complex mixtures varying in molecular weights with different end-groups and *O*-substitution patterns.<sup>17</sup> Synthetic glycoconjugates have emerged as an attractive alternative for the development of vaccines, since their well-defined structures have reproducible

physical, chemical, biological, and immunological properties.<sup>4</sup> In 2004, the large-scale synthesis of a glycoconjugate accomplished by the team of Vereze-Bencmou culminated in the world's first synthetic carbohydrate vaccine against *Haemophilus influenzae* type b in humans.<sup>18,19</sup> Since then, other projects aiming for the synthesis of many bacterial capsular polysaccharide and lipopolysaccharides fragments from many pathogens have been reported with the ultimate goal to generate vaccine candidates.<sup>20,21</sup> So far, synthetic vaccines against Shigellosis, *Streptococcus pneumoniae*, and *Staphylococcus aureus* are in pre-clinical or early clinical evaluation.<sup>22–26</sup> Previously, we described a synthetic teichoic acid fragment that mimics lipoteichoic acid from *E. faecalis* and is able to induce opsonic and protective antibodies that is a potential vaccine against unencapsulated CPS-A and CPS-B *E. faecalis* strains.<sup>27</sup> In the present work, we systematically evaluate different synthetic fragments that mimic the native DHG from *E. faecalis* Type 2 to define the best candidates for vaccine development against encapsulated *E. faecalis* that cannot be targeted using a teichoic-acid-based vaccine.

## RESULTS AND DISCUSSION

To discover a potent minimal DHG epitope, two series of DHG fragments were synthesized. The first was built up from [Galp-Glcp]-dimer repeats,<sup>28</sup> while the second set was composed of the alternative frameshifted [Glcp-Galp] dimers. Scheme 1 summarizes the syntheses of the glycan libraries. The first route of synthesis was based on the disaccharide building block **11**, containing temporary Fmoc-protection, obtained by coupling of furanoside donor **9**<sup>29</sup> and glycoside acceptor **10** (its synthesis described in the Supporting Information (SI)) with a 95% yield. Glycosylation of 3-trifluoroacetamidopropanol with donor **11** using a NIS/TfOH-system as a promotor resulted in spacer-containing disaccharide **12**. Temporary Fmoc-protection in **12** was removed by morpholine in DMF, which avoided benzoyl migration during deprotection<sup>28</sup> and obtained the desired acceptor in a 92% yield. The liberated hydroxyl group was subsequently glycosylated with donor **11**, resulting in tetrasaccharide **13** in a 85% yield. The removal of Fmoc-protection and glycosylation steps were repeated to form hexasaccharide **14** and octasaccharide **15** in good yields. The deprotection of oligosaccharides **12–15** was carried out by hydrolysis of benzylidene acetal in aqueous trifluoroacetic acid (TFA) in  $\text{CH}_2\text{Cl}_2$ , followed by the removal of acyl protections with MeONa in MeOH– $\text{H}_2\text{O}$  to give the first series of DHG-fragments (**1a–4a**).

The second set of oligomers was obtained using key disaccharide **16**. This building block has the obvious advantage that no benzoyl migration can take place from the Galp C(5) to the neighboring C(6) position during deprotection, as the  $\beta$ -D-Glcp-(1  $\rightarrow$  6)- $\beta$ -Galp linkage is constructed at the building block level (see the SI for full experimental details). Key building block **16** was equipped with a levulinic (Lev) ester as a temporary protecting group for the elongation of the oligomers, a silylidene ketal masking the C4- and C6-hydroxyls of the glucosyl moiety. The other alcohol groups were masked as benzoyl esters. We have previously used<sup>30</sup> a very similar protecting group pattern for a glucosamine synthon in the automated solid phase assembly of hyaluronic acid oligosaccharides. These syntheses revealed that the silylidene group not only is very resistant to acidic glycosylation conditions but also it endows the neighboring C(3)–OH with excellent reactivity.<sup>31</sup> First, an azidopropanol spacer was introduced on

Scheme 1. Synthesis of DHG Related Spacer-Armed Oligosaccharides and Their Conjugates with Biotin and BSA<sup>a</sup>

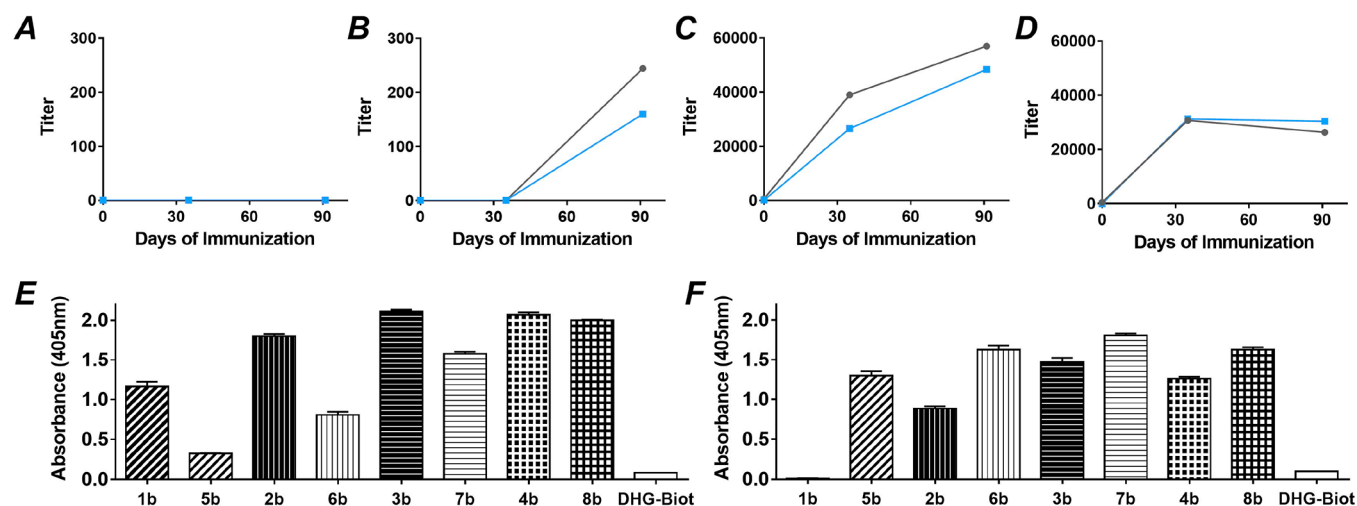
<sup>a</sup>Reagents and conditions: (a) TMSOTf, AW-300 MS, CH<sub>2</sub>Cl<sub>2</sub>, -70 → -10 °C; (b) NIS, TFOH, AW-300 MS, CH<sub>2</sub>Cl<sub>2</sub>, -40 → -5 °C; (c) morpholine (5 vol %), DMF; (d) first, TFA (90% aq), CH<sub>2</sub>Cl<sub>2</sub>; second, MeONa, MeOH, then H<sub>2</sub>O, 76–65%; (e) C<sub>6</sub>F<sub>5</sub>O-Biot, Et<sub>3</sub>N, DMF, room temperature (70–80%); (f) first, diethyl squarate, Et<sub>3</sub>N, EtOH, H<sub>2</sub>O; second, BSA, borate buffer (pH 9.0); (g) NIS, TMSOTf, CH<sub>2</sub>Cl<sub>2</sub>, -40 → -20 °C; (h) NH<sub>2</sub>NH<sub>2</sub>, PyH<sup>+</sup>AcO<sup>-</sup>/AcOH, 0 → +20 °C; (i) first, Et<sub>3</sub>N·3HF, THF, 80–85%; second, MeONa, MeOH; third, H<sub>2</sub>, Pd cat, 80–90%.

the disaccharide, using NIS and catalytic amounts of trimethylsilyl trifluoromethanesulfonate (TMSOTf) to activate the thioglycoside, delivering **17** in 70% yield. The resulting dimer was treated with hydrazine acetate to selectively unmask the Lev group, giving an acceptor in 95% yield. In a [2 + 2] coupling tetrasaccharide, **18** was obtained in 77% yield. Subsequent levulinoyl removal and glycosylation cycles allowed for the generation of the hexa- and octasaccharide **19** and **20** oligosaccharides in good yields. The deprotection of the four fragments **17**–**20** was carried out by cleavage of the silyl groups using a solution of Et<sub>3</sub>N·3HF in THF, followed by the removal of the benzoyl esters under Zemplén conditions, and finally, the reduction of the azide functionality through a hydrogenolysis reaction.

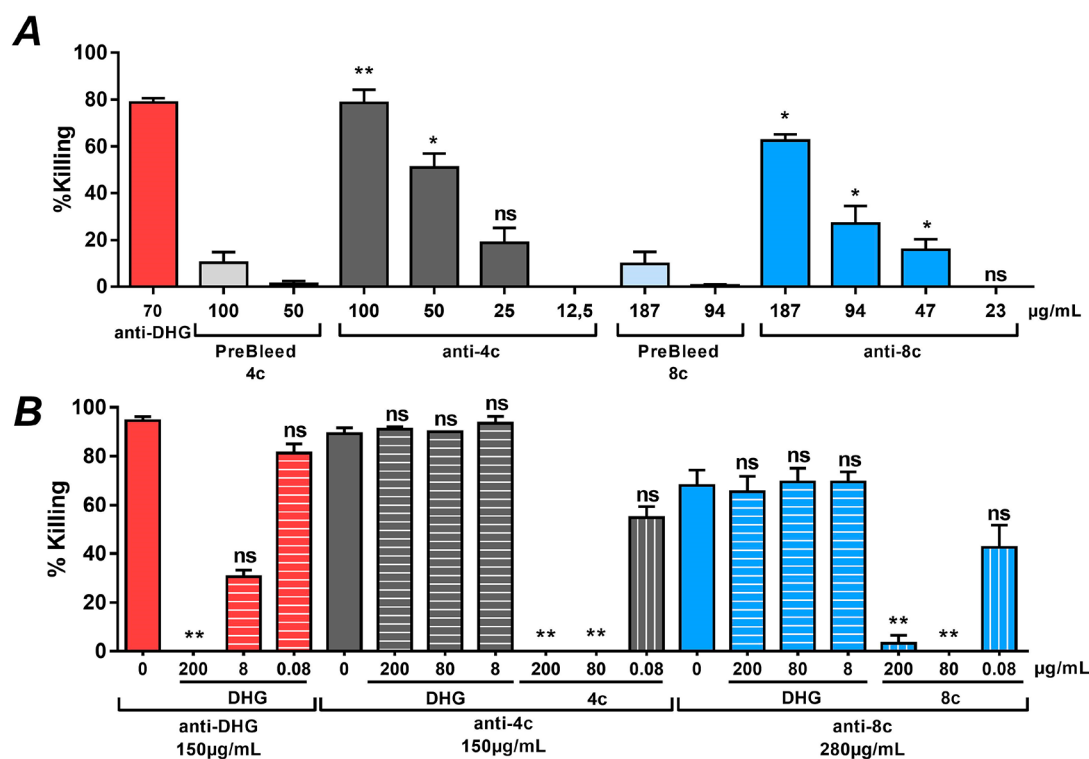
All synthesized DHG related oligosaccharides were equipped with a biotin group by treatment of aminopropyl glycosides **1a**–**8a** with biotin derivative C<sub>6</sub>F<sub>5</sub>O-Biot containing

a hexaethyleneglycol spacer, which is required for efficient spatial presentation of the oligosaccharide ligands in the glycoarray for biological recognition<sup>32</sup> after the immobilization on streptavidin coated plates (for examples, see refs 33–36). The structures of obtained biotinylated derivatives **1b**–**8b** were confirmed by HRMS data and by presence of the characteristic signals in <sup>1</sup>H NMR spectra (see Table S1 in the SI).

The conjugation of octasaccharides **4a** and **8a** with BSA was performed by the squarate method.<sup>37</sup> At the first step, the reaction of the parent oligosaccharides with diethyl squarate resulted in the monosubstituted adducts, which were then reacted with the free amino groups of BSA at pH 9, resulting in BSA-conjugates **4c** and **8c**. According to MALDI TOF mass spectrometry, the conjugates contained, on average, 18 octasaccharide antigenic ligands.

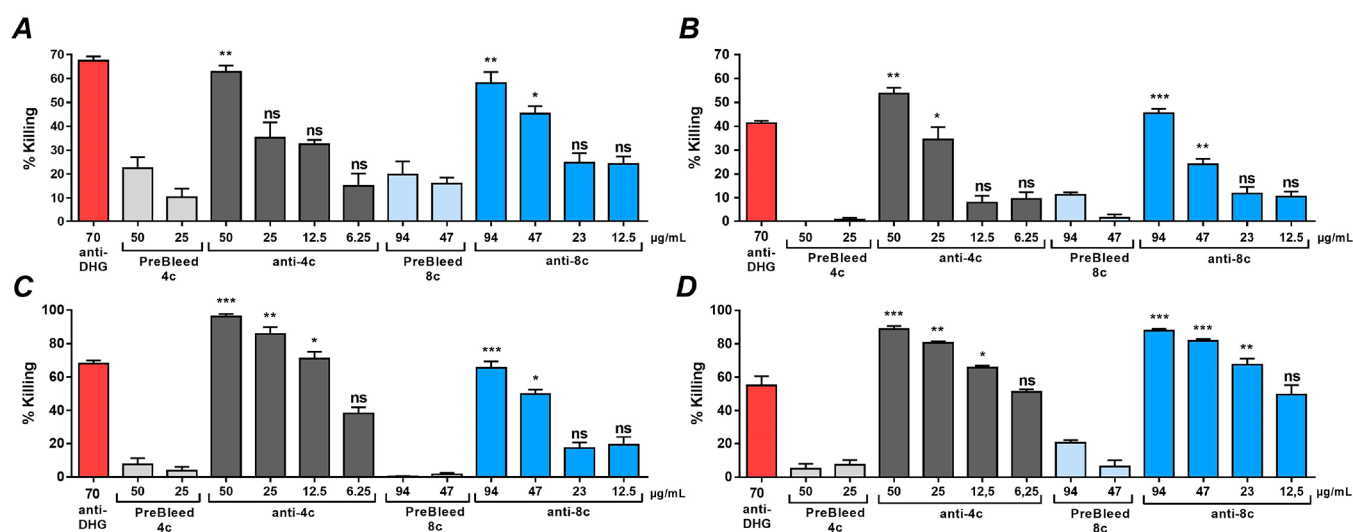


**Figure 2.** Specificity of sera raised against synthetic 4c and 8c conjugates. Rabbit sera obtained during immunization schedule were examined for specificity toward the different antigens. Streptavidin coated plates were coated with 1  $\mu$ g per well of (A) carrier protein BSA, (B) the native DHG, and synthetic (C) 4c and (D) 8c conjugates. Rabbit sera anti-4c (black) and anti-8c (blue) were plated in 2-fold serial dilutions, starting with a dilution of 50  $\mu$ g IgG/mL for each serum tested. (E) Anti-4c and (F) anti-8c at 1.25 and 2.4  $\mu$ g/mL, respectively, were also examined for their immunoreactivity toward synthetic DHG saccharides, dimers 1b and 5b, tetramers 2b and 6b, hexamers 3b and 7b, and octamers 4b and 8b. Biotinylated DHG (DHG-Biot) was used as the control to assess the preferred ability of the anti-DHG serum for the antigenic structures tested.



**Figure 3.** Analysis of opsonophagocytic killing activity of the antibodies. (A) Sera raised against 4c and 8c conjugates were evaluated in an opsonophagocytic assay to determine the opsonophagocytic killing activity of the sera against *E. faecalis* Type 2. Anti-4c (gray) and anti-8c (blue) sera were used at different concentrations, as shown in the x-axis. Serum raised against the native DHG polysaccharide was used as a positive control (red). (B) Specificity of the sera against the antigens was confirmed by inhibiting the opsonophagocytic killing activity with different amounts of the different antigens. Purified antibodies raised against synthetic 4c (gray) and 8c (blue) conjugates were used at a dilution yielding between 60 and 90% of killing and absorbed out with different amounts of native DHG (horizontal stripes) or synthetic DHG-conjugate (vertical stripes) as inhibitors. Serum raised against the native DHG polysaccharide was absorbed out with native DHG as a positive control. Sera without inhibitors were used as a positive control for opsonophagocytic killing. Effective opsonophagocytic (or inhibition of) killing in the anti-conjugate sera (anti-4c and anti-8c) was compared to pre-immune rabbit sera (PreBleed, in lighter color) by nonparametric Kruskal–Wallis test, followed by multiple comparisons using Dunn's post-test. Bars represent the mean of data, and the error bars represent the standard error of the mean (ns, nonsignificant, \* $P$  < 0.05, and \*\* $P$  < 0.01).





**Figure 4.** Opsonophagocytic killing activity of anti-4c and anti-8c sera against *E. faecalis* encapsulated bacterial strains. The antibodies raised against synthetic 4c (gray) and 8c (blue) conjugates were used at different concentrations. Evaluated *E. faecalis* CPS-C strains were (A) *E. faecalis* VS83 and (B) *E. faecalis* FA2-2 and *E. faecalis* CPS-D strains were (C) *E. faecalis* Type 5 and (D) *E. faecalis* Type 18. Effective opsonophagocytic killing in the anti-conjugate sera (anti-4c and anti-8c) was compared to pre-immune rabbit sera (PreBleed, in lighter color) by a nonparametric Kruskal–Wallis test, followed by multiple comparisons using Dunn’s post-test. Bars represent the mean of data, and the error bars represent the standard error of the mean (ns, nonsignificant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

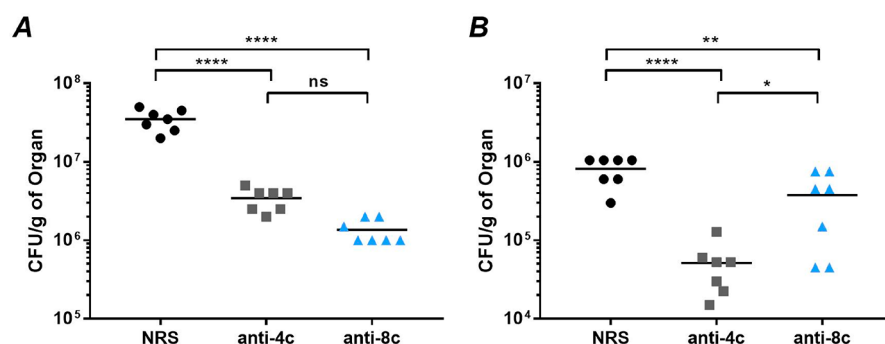
After synthesis and characterization, the biotinylated compounds were evaluated for their ability to immunoreact with serum raised against native DHG in an ELISA using streptavidin coated plates (see Figure 1B). A clear length dependence was observed for the synthetic oligosaccharides, with the longer synthetic saccharides (i.e., octamers 4b and 8b and hexamers 3b and 7b) showing better immunoreactivity with anti-DHG serum than shorter ones (i.e., tetramers 2b and 6b and dimers 1b and 5b). This correlates with the previously described results, in which synthetic saccharides mimicking native polysaccharides conjugated to a carrier protein are good antigens that elicit opsonic and protective antibodies.<sup>27,38,39</sup> Here, we have shown that the synthesized hexa- and octa-oligosaccharides elicit the highest immunogenicity and may therefore partially mimic the immunogenic properties of the native DHG. Considering that the octasaccharides are not more difficult to synthesize than the hexasaccharides, and may better adopt the conformation of the native polysaccharide,<sup>40</sup> we decided to use the octasaccharides 4a and 8a for further immunological studies and evaluated their candidacy for vaccine development against enterococcal infections.

The conjugates 4c and 8c were used to immunize rabbits, and bleeds were taken at different time points during immunization. The resulting anti-4c and anti-8c sera were analyzed for specific IgG against native DHG, 4c, 8c, and BSA (see Figure 2A–D). No significant antibody levels were detected against the carrier protein BSA in the sera raised against conjugates 4c and 8c (see Figure 2A). Interestingly, low titers were raised against the native polysaccharide (see Figure 2B). This observation may be explained by the fact that the substitutions on the backbone structure of the native DHG polysaccharide mask the epitopes targeted by sera raised against the unsubstituted synthetic oligosaccharides 4c and 8c. Both sera exhibited very high titers against the conjugates (see Figure 2C,D), with the response against structure 4c being higher than that against 8c. These differences in immune responses may be explained by the monosaccharides at the terminus of the octasaccharides 4c and 8c being either a “non-

self” galactofuranose, which is foreign residue for mammalian glycans, or a “self” glucose residue typical for host glycolyx. Especially in the case of relatively short (with respect to native polysaccharides) synthetic oligosaccharides displaying less internal epitopes, the nature of the terminal sugar often plays an important role in recognition by antibodies.<sup>24,41,42</sup> To overcome the disparity in the immune response of the rabbits used for the immunization with the two conjugates, in subsequent experiments, the anti-8c serum was used 1.87 times more concentrated than the anti-4c serum.

The generated immune sera were further evaluated against the synthetic DHG saccharide library (see Figure 2E,F). As shown in Figure 2E, anti-4c immunoreacted preferentially with the structures with a terminal Gal $\beta$ -(1  $\rightarrow$  3)-unit at the left end of the chain. Both dimer fragments 1b and 5b were recognized by this serum with better immunoreactivity. However, the anti-8c serum only recognized the dimer fragment 5b as being the smallest epitope capable of binding. Also for this serum, a slight preference in binding was observed for the Glcp-Galf-repeating unit structures, although the preference was less pronounced in comparison to that of the anti-4c serum. Both sera showed no immunoreactivity toward biotinylated DHG at the concentration used, but at higher concentrations of sera (150  $\mu$ g IgG/mL), an immunoreactivity similar to the saccharide library (2.0 U.A.) was observed toward Biotinylated DHG (see Figure S2).

Subsequently, opsonophagocytic killing activities of anti-4c and anti-8c were assessed against *E. faecalis* Type 2 strain, from where DHG was purified and structurally elucidated.<sup>10,16</sup> The pre-bleeds and terminal bleeds of the rabbits were evaluated at different concentrations to assess dose dependent killing activity of the target strain (Figure 3A). We observed that both terminal sera mediated opsonophagocytic killing of the bacterial strain at relatively low IgG concentrations (100  $\mu$ g/mL for anti-4c and 187  $\mu$ g/mL for anti-8c) that are comparable to previously reported antibodies raised against other Gram-positive bacterial capsular polysaccharides.<sup>43–45</sup> The lack of opsonophagocytic killing in samples with the pre-



**Figure 5.** Mouse sepsis model. Mice were passively immunized with the sera raised against 4c (gray squares) and 8c (blue triangles) conjugates and challenged with *E. faecalis* Type 2. After 48 h of challenge, mice were sacrificed and their livers and kidneys were removed to assess viable counts. Panels (A) and (B) show the resulting viable counts in mice livers and kidneys challenged with *E. faecalis* Type 2, respectively. Each point represents the bacterial counts from a single mouse. Bars indicate the median CFU/g of organ for the group. Statistical analysis was done by one-way analysis of variance (ANOVA) with a Dunnett's correction post-test comparing between the animals immunized with the antibodies raised against the DHG-protein conjugates and control animals immunized with normal rabbit serum (NRS, black circles). Horizontal bars represent geometric means (ns, nonsignificant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

immune sera demonstrates that the activities observed for the terminal bleeds are mainly due to antibodies raised by immunization with the conjugates. Moreover, control anti-DHG sera mediated opsonophagocytic killing at a slightly lower IgG concentration (i.e., 70  $\mu\text{g}$  IgG/mL). This could be attributed to the fact that the native DHG possesses a more varied epitope repertoire because of its larger structure having different conformations and decorations, which may contribute to promote a better immune response. Also, the much higher antibody titers in anti-DHG sera against native polysaccharide (i.e., 6400 for anti-DHG (results not shown), 244 for anti-4c and 160 for anti-8c in Figure 2) could explain the observed differences in the opsonic activity.

Specificity of the opsonic antibodies toward the target antigens was confirmed by an opsonophagocytic inhibition assay OPIA (see Figure 3B). Purified antibodies were used to avoid interference of pre-existing antibodies or any other components in sera that may interact during the pre-absorption of the antibodies with the antigens for the OPIA.<sup>46,47</sup> The activity of control anti-DHG was completely abolished when pre-incubated with a high concentration of native DHG and was partly restored when the concentration of the DHG was lowered. Similarly, the 4c and 8c conjugates bound to the anti-4c and anti-8c antibodies, thereby inhibiting killing of the target strain. For both anti-conjugate sera, no inhibitory effect was observed when pre-incubation with native DHG was carried out. This could be explained by the microheterogeneities and substituents present in the native DHG that may have hindered the epitopes that anti-4c and anti-8c target since the conjugates mimic the structure of the native DHG backbone.<sup>42,48</sup>

After we demonstrated that the conjugates were able to elicit specific and opsonic antibodies against the target strain, we proceeded to investigate their cross-reactivity against different encapsulated *E. faecalis* strains. We selected different CPS-C (*E. faecalis* VS83 and FA2-2) and CPS-D (*E. faecalis* Type 5 and Type 18) strains that are susceptible to antibodies raised against the native DHG isolated from *E. faecalis* Type 2.<sup>10,12</sup> Both anti-4c and anti-8c sera were able to mediate opsonic killing of all the strains tested (see Figure 4). Overall, both sera were more effective against CPS-D than CPS-C strains, exhibiting killing percentages ranging from 45 to 60 and 70 to 90%, respectively. Interestingly, *E. faecalis* CPS-D strains

were more sensitive to anti-4c and anti-8c than to anti-DHG serum. These results could be explained due to the microheterogeneity in the DHG of the different evaluated serotype strains.<sup>10,12,49</sup> Possibly, DHG in CPS-D strains is less densely substituted (with acetyl and lactic acid groups) than the DHG from the CPS-C serogroup, making its DHG backbone more accessible to the raised antibodies. More structural data for the DHG of these *E. faecalis* serotypes is required to confirm this hypothesis.

Finally, the *in vivo* protective potential of the synthetic oligomer conjugates was evaluated in a mouse sepsis model (see Figure 5). Mice were passively immunized with normal rabbit serum (NRS), anti-4c, or anti-8c and were challenged with  $1 \times 10^8$  CFU *E. faecalis* Type 2/mouse. Two days after bacterial challenge, animals were sacrificed, their organs were aseptically removed and homogenized, and the bacterial counts were enumerated by serial dilutions and plating. Both sera were able to significantly reduce the colony counts in livers and kidneys of the mice in comparison to NRS control. Differences in the protection conferred between the sera in mice livers were not statistically different. However, in mice kidneys, anti-4c sera significantly conferred a better protection than anti-8c sera. This correlates with the fact that the 4c antigen is able to raise a better immune response, possibly because of the “non-self” galactofuranose residue at its terminus, as previously shown by the antibody titers and opsonophagocytic killing activities that are slightly superior to those exhibited by anti-8c sera. In addition, differences in the protection conferred by the two sera may be explained by variations in polysaccharide expression *in vivo*. It has been previously demonstrated in other Gram-positive pathogens, including *S. aureus*, that micro-environments (i.e., site of infection), bacterial strain, and/or stage of infection alter the polysaccharide composition in the pathogen.<sup>50–53</sup> Nevertheless, more comprehensive animal studies should be conducted to confirm this hypothesis.

In conclusion, we have generated a library of synthetic DHG fragments, ranging in length from dimers to octasaccharides, built up from either [Galf-Glcp] or [Glcp-Galf]-disaccharide repeats, which was used to identify synthetic DHG immunogens. The octasaccharides 4a and 8a were conjugated to BSA and used for immunization. The antibodies raised with the synthetic conjugates were able to mediate the opsonic killing of encapsulated *E. faecalis* CPS-C and CPS-D strains

expressing DHG. Even though the native DHG capsule is decorated with acetyl and lactic acid substituents, the serum raised was capable of recognizing the native DHG, indicating that our glycoconjugates are appropriate immunogens to raise specific and opsonic antibodies. The role of lactic acid and *O*-acetyl substituents in *E. faecalis* DHG should be evaluated in future studies to determine whether these substituents constitute nonessential or protective epitopes, as has been described for other bacterial pathogens.<sup>54–56</sup> According to the antibody titers, for opsonophagocytic killing activity and protection *in vivo* conferred by sera against *E. faecalis* encapsulated strains, the 4c antigen showed a slightly better immune response. This may be due to the fact that octasaccharide 4c with the [Gal $\beta$ -Glc $\alpha$ ]-repeating unit presents a “non-self” sugar at its terminus, while 8c has a “self” glucopyranose at this position, leading to inferior recognition by the immune system. Altogether, our results show that these synthetic structures mimicking DHG are promising vaccine candidates against the encapsulated *E. faecalis* strains that are a major concern in the clinical setting. These antigens could be used in combination with, for example, well-defined teichoic acid fragments and immunogenic proteins to generate broad coverage multivalent conjugate vaccines to fight these important nosocomial pathogens.

## METHODS

**Bacterial Strains and Culture Conditions.** In this study, the Japanese prototype strain first described by Maekawa et al. *E. faecalis* Type 2 was used.<sup>57</sup> For the cross-reactivity tests, we used the vancomycin-resistant bloodstream isolate *E. faecalis* V583,<sup>58</sup> the derived strain from a patient isolate *E. faecalis* FA2-2,<sup>59</sup> and the Japanese prototype strains *E. faecalis* Type 5 and *E. faecalis* Type 18.<sup>57</sup> For the opsonophagocytic assays, strains were grown in Tryptic soy broth (Carl Roth) at 37 °C without agitation from an overnight grown plate of Tryptic soy agar, freshly prepared from the –80 °C bacterial stock. For polysaccharide purification, *E. faecalis* Type 2 was grown in Columbia broth (Becton Dickinson) supplemented with 2% glucose at 37 °C without agitation until an optical density of 0.8 at 600 nm was reached.

**Native DHG Purification and biotinylation.** DHG of *E. faecalis* was isolated as previously described.<sup>60</sup> Briefly, a bacterial strain was used to inoculate Columbia broth (Becton-Dickinson) supplemented with 2% glucose and incubated at 37 °C without agitation until an optical density at 600 nm of 0.8 was reached. Bacterial cells were pelleted by centrifugation at 8000 rpm for 30 min at 4 °C and were resuspended with Tris-sucrose buffer (10 mM Tris-HCl, 25% sucrose, pH 8.0). After digestion of the bacterial cell wall using lysozyme and mutanolysin at 500 and 10  $\mu$ g/mL, respectively, the cell debris and insoluble components were removed by centrifugation at 8000 rpm for 30 min at 4 °C. Then, the supernatant was treated with 100  $\mu$ g/mL of nucleases and proteases and was dialyzed against deionized water prior to freeze-drying. Subsequently, the crude of polysaccharide was fractionated by size exclusion chromatography in a 1.6 cm  $\times$  90 cm sephacryl S-400 column (GE Healthcare) equilibrated in 50 mM Tris buffer (pH 7.2) buffer, using a flow rate of 0.5 mL/min. Eluted fractions were monitored by UV absorption at 214 and 254, with a differential refractometer (Gilson), and were analyzed for sugar, methylpentose, *O*-acetyl group, and phosphorus content by different colorimetric assays.<sup>61–65</sup> Fractions positive for sugar and *O*-acetyl and negative for

phosphorus and methylpentose were combined, concentrated, and desalted with a 3 kDa Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) and were subjected to further purification with two in-series 5 mL HiTrap Q FF sepharose anion-exchange columns (GE Healthcare). For elution, a linear gradient from 0 to 1 M NaCl in 10 mM Tris buffer (pH 7.2) was used. Bound carbohydrate eluted from the column at 0.45 M NaCl, also positive for sugar and *O*-acetyl colorimetric assay and negative for phosphorus and methylpentose groups, were combined, concentrated, and desalted with a 3 kDa Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) prior to lyophilization. <sup>1</sup>H NMR of the purified DHG was recorded at 500 MHz in deuterated oxide D<sub>2</sub>O to determine its purity for subsequent experiments (see the [Supporting Information Figure S1](#)).

The biotinylation of DHG was performed as described by Zhang et al.<sup>45</sup> Native DHG (1 mg) was dissolved in LPS-free water (cell culture grade; HyClone) at 5 mg/mL and mixed under vortexing with 1 mg of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate at 100 mg/mL in acetonitrile (Sigma-Aldrich). After 30 s, the pH was increased to 8 by adding 7  $\mu$ L of 0.2 M triethylamine (Sigma-Aldrich). Exactly after 2.5 min, 1 mg of Amine-PEG3-Biotin (Sigma-Aldrich) at 20 mg/mL in water was added. The mixture was incubated for 4 h at room temperature, and 25 mM of glycine was used to quench the reaction. Remaining biotin was removed by diafiltration using 3 kDa Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) against 10 mM Tris, pH 7.2. The concentrations of polysaccharide and biotin in the biotinylated DHG were quantified by anthrone assay and the biotin quantification kit (Pierce), respectively.

**Synthesis of Saccharides Mimicking DHG and Conjugation.** All synthetic procedures for the preparation of oligosaccharides 1a–8a, their conjugates with biotin (1b–8b) and BSA (4c, 8c), characterizations for new compounds, and copies of <sup>1</sup>H and <sup>13</sup>C NMR spectra are provided in the [Supporting Information](#).

**Rabbit Immunizations.** A New Zealand white rabbit was immunized with purified DHG from *E. faecalis* Type 2 as described elsewhere (anti-DHG).<sup>9</sup> For 4c and 8c conjugates, a New Zealand white rabbit was immunized per antigen by two subcutaneous injections of 10  $\mu$ g of conjugate given 1 week apart from each other. An intravenous (iv) injection of 5  $\mu$ g of conjugate was given 7 days after, followed by two more iv injections of 5  $\mu$ g of conjugate 2 days apart from each other. On day 35, the test bleed was taken, and 7, 14, 28, and 42 days later, iv boosts of 5  $\mu$ g of antigen were performed. On day 91, a final bleed of each rabbit was taken. All sera were heated at 56 °C for 30 min to inactivate the complement components. Pre-immunization sera samples were taken twice from each rabbit 1 week apart from the first immunization as a control in the assays. Immune sera raised against conjugates were named anti-4c and anti-8c, respectively.

**Quantification of Rabbit IgGs.** Total rabbit IgG in both purified and unpurified anti-DHG, anti-4c, and anti-8c sera were quantified as previously described by Salauze et al.<sup>66</sup> Nunc-immuno Maxisorp MicroWell 96-well plates were coated with 0.1  $\mu$ g per well of unlabeled antirabbit IgG (Sigma-Aldrich) in 0.2 M carbonate–bicarbonate coating buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Next day, wells were washed three times with 200  $\mu$ L of washing buffer (WB: 0.9% sodium chloride supplemented with 0.1% Tween 20) and



blocked with 200  $\mu$ L of blocking buffer (BB: 3% BSA (Carl Roth)) in 1  $\times$  PBS at room temperature (RT) for 2 h. Meanwhile, dilutions of standard rabbit IgG (Sigma) ranging from 31.2 to 0.12 ng/mL as well as purified and unpurified antibodies in dilutions ranging from 1:10 000.000 to 1:50 000.000 were prepared in a blocking buffer. After incubation, wells were washed three times with 200  $\mu$ L of WB and 100  $\mu$ L in triplicate of samples, and standard dilutions were plated. Incubation was carried out for 2 h at RT, and the plates were then washed three times with 200  $\mu$ L of WB. As the secondary antibody, 100  $\mu$ L of alkaline-phosphatase-conjugated antirabbit IgG produced in goat (Sigma) diluted 1:1000 was used. Incubation was carried out for 2 h at RT. After, the plates were washed four times with 200  $\mu$ L of WB. For detection, 100  $\mu$ L of *p*-nitrophenyl phosphate (Sigma-Aldrich) at 1 mg/mL in glycine buffer (0.1 M glycine, 1 mM  $\text{MgCl}_2$ , and 1 mM  $\text{ZnCl}_2$ , pH 10.4) were used. After 30 min of incubation at RT, the reaction was stopped by adding 50  $\mu$ L of 3 M sodium hydroxide and absorbance was measured at 405 nm. Antibody concentrations were calculated against the IgG calibration curve generated with standard rabbit IgG dilutions.

**Screening of the Synthetic DHG Saccharides by ELISA.** Anti-DHG serum specificity against different synthetic disaccharides mimicking the native DHG backbone was evaluated by ELISA. High capacity streptavidin coated plates (Thermo Fischer Scientific) were washed three times with tween-BSA buffer (TBB: 1 $\times$ TBS, 0.1% BSA, and 0.05% Tween 20). Then, the wells were coated in triplicate with 1  $\mu$ g of synthetic saccharides dissolved in TBB. Plates were incubated for 2 h at 4  $^{\circ}\text{C}$ , washed three times with 200  $\mu$ L of TBB, and incubated with 100  $\mu$ L of anti-DHG serum at the specified concentration for 2 h at RT with gentle shaking. Wells were washed three times with 200  $\mu$ L of TBB and incubated for 30 min at RT with a 100  $\mu$ L of alkaline-phosphatase-conjugated antirabbit IgG produced in goat at a 1:1000 dilution (Sigma). As a substrate, 100  $\mu$ L of *p*-nitro phenyl phosphate (Sigma) was used at 1 mg/mL in glycine buffer. Plates were incubated at RT in the dark for 40 min; the reaction was stopped by adding 50  $\mu$ L of 3 M sodium hydroxide, and the absorbance was measured at 405 nm.

**Measurement of Antigen Specific Antibodies in Rabbit Sera by ELISA.** Nunc-immuno Maxisorp 96-well plates were coated with 1  $\mu$ g/well of either BSA, DHG, 4c, or 8c in 0.2 M sodium carbonate/bicarbonate buffer (60 mM  $\text{Na}_2\text{CO}_3$ , 140 mM  $\text{NaHCO}_3$ , pH 9.4) and incubated overnight at 4  $^{\circ}\text{C}$ . After incubation, wells were washed three times with 200  $\mu$ L of PBS-Tween buffer (PTB: PBS, 0.05% Tween 20, pH 7.4) and blocked for 1 h with 100  $\mu$ L of blocking buffer (PBS, 3% BSA, pH 7.4) at 37  $^{\circ}\text{C}$ . Meanwhile, 2-fold serial dilutions of rabbit sera were prepared, starting with a dilution of 100  $\mu$ g IgG/mL for each serum tested. Later, wells were washed twice with 200  $\mu$ L of PTB. After, 100  $\mu$ L of the rabbit sera were plated in triplicates and incubated 1 h at 37  $^{\circ}\text{C}$ . Then, wells were washed three times with 200  $\mu$ L of PTB prior to 1 h incubation with 100  $\mu$ L of alkaline-phosphatase-conjugated antirabbit IgG produced in goat at a 1:1000 dilution (Sigma). After, wells were washed four times with 200  $\mu$ L of PTB prior to 30 min incubation with 100  $\mu$ L of the *p*-nitro phenyl phosphate substrate (Sigma) at 1 mg/mL in glycine buffer. The reaction was stopped by the addition of 50  $\mu$ L of 3 M NaOH, and absorbance was measured at 405 nm. Serum IgG titers were calculated as follows: For each sample, a plot of the OD value against the reciprocal of the dilution (i.e.,

$\log_{10}[\text{dilution factor}]$ ) was used to calculate the intercept of an absorbance of 1 for each test; this was taken as the ELISA end point titer. The value extrapolated from the standard curve was then multiplied by the inverse of that dilution to generate the final inverse titer.

**Purification of Rabbit Antibodies.** Rabbit sera raised against DHG and synthetic conjugates were purified with a rProtein A GraviTrap column (GE Healthcare) according to manufacturers' instructions. In brief, 10 mL of binding buffer (20 mM sodium phosphate, pH 7.0) were used to equilibrate the column. Rabbit serum (2 mL) was supplemented with 100  $\mu$ L of 20 $\times$  binding buffer (0.4 M sodium phosphate, pH 7.0) before loading onto the column. The column was washed with 15 mL of 1 $\times$  binding buffer, and seven fractions of 1 mL each were collected after eluting the antibodies by the addition of 10 mL of glycine buffer (0.1 M glycine-HCl, pH 2.7). Immediately after elution, fractions were mixed with 100  $\mu$ L of 1 M Tris-HCl, pH 8.0, to preserve activity of acid-labile IgGs. Protein positive fractions containing the purified IgGs were collected and diafiltrated with PBS in a 30 kDa Amicon ultra-0.5 mL device (Merck Millipore).

**Opsonophagocytic Assay (OPA) and Opsonophagocytic Inhibition Assay (OPIA).** An *in vitro* opsonophagocytic assay was performed as described elsewhere.<sup>60</sup> For inhibition of opsonophagocytic activity, purified antibodies from the immune sera were inhibited by the corresponding synthetic conjugate or native DHG, accordingly. Concentrations ranging from 0.08 to 200  $\mu$ g/mL of the sample were incubated overnight at 4  $^{\circ}\text{C}$  with an equal volume of a purified antibody (at 100  $\mu$ g/mL of anti-DHG, 300  $\mu$ g/mL of anti-4c, and 1.5 mg/mL of anti-8c). After incubation, the mixture of inhibitor/serum was used as a source of antibodies in an OPA as described above.

**Mouse Sepsis Model: Passive immunization.** The sepsis mouse infection experiment was performed as described previously with some modifications.<sup>67–69</sup> Briefly, BALB/c mice (weight, 20–25 g; Harlan) were split in three groups of seven mice and passively immunized as follows: intraperitoneally injected three times with 200  $\mu$ L of either normal rabbit serum (NRS), anti-4c, or anti-8c at 48 and 24 h prior and 4 h after the bacterial challenge. Bacterial inoculum was prepared by growing *E. faecalis* Type 2 in brain heart infusion broth (Sigma) supplemented with 40% heat-inactivated horse serum (Sigma) until the stationary phase was reached. After, bacterial cells were harvested by centrifugation, and the resulting pellets were resuspended in sterile PBS to a concentration of  $10^9$  CFUs/mL. Prior to the bacterial challenge with 100  $\mu$ L of the bacterial suspension, mice were anesthetized by intraperitoneal injection of 100 mg/g ketamine (Merial) and 12 mg/g xylazine (Bayer). The mice were monitored twice per day before they were sacrificed by cervical dislocation 48 h after the bacterial challenge. Kidneys and livers were aseptically removed, weighed, and homogenized in PBS for 120 s at a high speed in a stomacher (Pbi International). Serial dilutions were plated onto Enterococcus selective agar (Fluka Analytical) to determinate the colony-forming unit (CFU) numbers.

**Statistics.** For statistical analysis, a Prism version 7.00 (GraphPad) was used. The percentage of opsonophagocytic killing and absorbance in whole-cell ELISA was expressed as the geometrical mean with the standard errors of the mean. For OPA and OPIA, statistical significance was determined by nonparametric Kruskal–Wallis test followed by multiple comparisons using Dunn's post-test. *In vivo* experiments,

results were subjected to statistical analysis by using one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test. *P* values <0.033 were considered statistically significant.

**Ethics Statement.** Rabbits were housed, immunized, and sampled by Biogenes (Berlin, Germany), in accordance with national and international animal welfare regulations. Rabbit immunizations at this facility were under approval from NIH/OLAW Animal Welfare Assurance (ID #A5755-01). Mouse experiments were conducted under a protocol approved by the Institutional Animal Use and Care Committee at Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli IRCCS and authorized by Italian Ministry of Health (Protocol number: 1F295.37, 11/05/2017; Authorization number: 903/2017-PR, 11/05/2017) according to the Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00063>.

Discussions of materials and methods used, synthetic procedures, and characterization data, schemes of synthetic pathways, table of synthesis yields and HRMS data, and figures of NMR spectra, specificity of sera, and MALDI-TOF spectra (PDF)

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## Author Contributions

▲D.L., F.R.-S., D.A.A., and J.E. contributed equally. D.L., F.R.-S., E.K., J.H., C.M., R.T., and M.S. conceived and designed the *in vitro* and *in vivo* experiments. J.E., J.D.C.C., V.B.K., D.A.A., and N.E.N. performed the synthetic experiments. D.L., F.R.-S., J.E., J.D.C.C., V.B.K., D.A.A., N.E.N., and J.H. cowrote the paper. D.L., F.R.-S., E.K., V.B.K., D.A.A., N.E.N., J.E., J.D.C.C., and J.H. participated in review and editing.

## Notes

The authors declare no competing financial interest.

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