Engineering the *Campylobacter jejuni* N-glycan to create an effective chicken vaccine

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*Campylobacter jejuni* is a predominant cause of human gastroenteritis worldwide. Source-attribution studies indicate that chickens are the main reservoir for infection, thus elimination of *C. jejuni* from poultry would significantly reduce the burden of human disease. We constructed glycoconjugate vaccines combining the conserved *C. jejuni* N-glycan with a protein carrier, GlycoTag, or fused to the *Escherichia coli* lipopolysaccharide-core. Vaccination of chickens with the protein-based or *E. coli*-displayed glycoconjugate showed up to 10-log reduction in *C. jejuni* colonization and induced N-glycan-specific IgY responses. Moreover, the live *E. coli* vaccine was cleared prior to *C. jejuni* challenge and no selection for resistant campylobacter variants was observed. Analyses of the chicken gut communities revealed that the live vaccine did not alter the composition or complexity of the microbiome, thus representing an effective and low-cost strategy to reduce *C. jejuni* in chickens and its subsequent entry into the food chain.

Campylobacter infections (primarily *C. jejuni* or *C. coli*) are among the most prevalent cause of human gastroenteritis worldwide1,2. Since *C. jejuni* is a common member of the chicken intestinal microbiome, poultry are major sources for human infection that results in the development of watery diarrhea, hemorrhagic colitis and in some cases reactive arthritis, Reiter’s syndrome, irritable bowel syndrome, and Guillain-Barré syndrome3,4. Thus, reducing *C. jejuni* at the source would significantly decrease the risk of human exposure and have a tremendous impact on food safety and public health.

Key prerequisites for antigens to be considered as vaccine candidates are immunogenicity and surface exposure. Attenuated campylobacter whole cell vaccines and nanoparticle encapsulated crude outer membrane protein lysates have been tested, but demonstrated limited protection5,6. More rational approaches included the use of specific protein antigens either purified, DNA-based or delivered by attenuated Salmonella strains. These include the flagellin subunit FlaA7,8, the outer membrane protein MOMP9, the adhesin Pbe110, the multidrug efflux pump component CmeC11, the ferric enterobactin receptor CfrA, the lipoproteins CjaA and CjaC (mediating amino acid transport)12, among others13–19. Although target-specific antibody responses were induced in most cases, the response provided either limited protection (FlaA-LTB20; rCmeC21; CjaD22 Dps23), was targeted against conformationally variable epitopes (MOMP)24,25, was not cross-protective (FlaA)26,27 or the results were highly variable (CjaA or CjaA-TetC)28–31 dependent on the model system or the route of administration. More recently egg yolk produced α-CadF, α-MOMP, and α-CmeC IgYs were suggested to be potentially useful as passive immunotherapeutics32, but their application did not result in a reduction of campylobacter colonization in chickens33.

Carbohydrates represent another class of biomolecules that have been successfully used for the generation of human glycoconjugate vaccines, but are currently not commercially available for animals34. *C. jejuni* is rich in surface carbohydrates including O- and N-linked glycoproteins35,36, capsular polysaccharides (CPS), and lipooligosaccharides (LOS); and studies using campylobacter CPS structures as antigens are showing promise in vaccine trials for human use37–39. However, since 47 different CPS serotypes have been identified for *C. jejuni* so far, the number of CPS types needed to achieve broad coverage against the most prevalent strains of *C. jejuni* needs to be determined and monitored for shifting populations37. Similarly, the variability in LOS and O-glycan

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structures limit the use of those carbohydrates as potential antigens. We were therefore interested in evaluating the use of the \textit{C. jejuni} N-glycan as a vaccine candidate in chickens. The \textit{C. jejuni} N-glycan is a heptasaccharide (GalNAc-\(\alpha_1,4\)-GalNAc-\(\alpha_1,4\)-[Glc-\(\beta_1,3\)-1,3]GalNAc-\(\alpha_1,4\)-GalNAc-\(\alpha_1,4\)-GalNAc-\(\alpha_1,4\)-[2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose, GalNAc is N-acetylgalactosamine and Glc is glucose]40,41 that is common to all \textit{C. jejuni} and \textit{C. coli} isolates tested35,36. The N-glycan is constitutively expressed, added to multiple periplasmic and membrane proteins, protects the bacteria against proteolytic attack, is immunogenic in rabbits and humans, plays a role in innate and adaptive immunity, and is required for the colonization of mice and chickens, adherence and invasion of human epithelial cells and natural competence35,42–44. Moreover, the \textit{C. jejuni} protein glycosylation (\textit{pgl}) genes are transferable into heterologous hosts like \textit{E. coli}40, to produce glycoproteins for biotechnological applications44.

Here we present two vaccine strategies. For the first approach, we created a glycoprotein that is comprised of a natural occurring \textit{C. jejuni} peptide (GlycoTag, GT) that contains 9 perfect repeats of the bacterial N-glycosylation sequon (D/E-X\(1\)-N-X\(2\)-S/T, where X\(1\) and X\(2\) can be any amino acid but proline45) and is readily modified with up to 9 \textit{C. jejuni} N-glycans (Fig. 1A). In the second approach, a whole cell surface display system was used to fuse the N-glycan structure to the outer core of the \textit{E. coli} lipopolysaccharide (LPS), replacing the natural O-antigen. Birds vaccinated with the GlycoTag-based or \textit{E. coli} cell-surface exposed N-glycans showed N-glycan-specific immune responses and significant reductions in \textit{C. jejuni} colonization levels after campylobacter challenge. The \textit{E. coli} live vaccine was self-limiting and did not affect the composition of the chicken gut community thus providing an inexpensive and effective vaccination strategy to reduce \textit{C. jejuni} in poultry.

**Results**

**Expression of the protein-based \textit{C. jejuni} N-glycan vaccine.** To create an effective protein glycoconjugate for the one-pot synthesis of N-glycoproteins in \textit{vivo}, we identified a novel campylobacter-derived N-glycan acceptor peptide, GlycoTag, located in the N-terminus of Cj1433c. GlycoTag contains 9 perfect repeats of the amino acid sequence KIDLNT including the bacterial N-glycosylation sequon (D/E-X\(1\)-N-X\(2\)-S/T, where X\(1\) and X\(2\) can be any amino acid but proline) and is readily modified with up to 9 \textit{C. jejuni} N-glycans when GlyoTag is fused to ToxC (Fig. 1A). In the second approach, a whole cell surface display system was used to fuse the N-glycan structure to the outer core of the \textit{E. coli} lipopolysaccharide (LPS), replacing the natural O-antigen. Birds vaccinated with the GlycoTag-based or \textit{E. coli} cell-surface exposed N-glycans showed N-glycan-specific immune responses and significant reductions in \textit{C. jejuni} colonization levels after campylobacter challenge. The \textit{E. coli} live vaccine was self-limiting and did not affect the composition of the chicken gut community thus providing an inexpensive and effective vaccination strategy to reduce \textit{C. jejuni} in poultry.
Expression and validation of the whole cell E. coli-C. jejuni N-glycan vaccine. To present the C. jejuni N-glycan on the E. coli cell surface, we fused the heptasaccharide to the outer LPS-core in an E. coli K12 O-antigen polymerase (wzy::kan) mutant background to avoid potential polymerization of the N-glycan structure (Fig. 2A). E. coli K12 does not produce endogenous O-antigen (O16) due to a naturally occurring mutation in the wbbL (rhamnosyltransferase) gene. Western blotting with C. jejuni N-glycan-specific antiserum (R1-4) confirmed the formation of the LPS-core-N-glycan molecule is indicated by an arrow. Molecular weight markers (in kDa) are indicated on the left. FACS analysis of 2 × 10⁴ cells of E. coli wzy::kan (pACYC184 (pglmut)) in light grey and E. coli wzy::kan (pACYC184) in dark grey. Fluorescent intensity is shown on the x-axis, cell counts (arbitrary units) are shown on the y-axis. (D) The sequence of the LPS core N-glycan fusion product is shown. N-glycan-derived monosaccharide residues that could be assigned by NMR are shown. Capital letters refer to residues as outlined in Supplementary Table 2. (E) NMR spectrum of the purified LPS core-C. jejuni-N-glycan compound. Correlations from anomeromic protons (as indicated) are shown. Green, COSY; red, TOCSY; black, ROESY.

Figure 2. The E. coli cell surface display-based C. jejuni N-glycan vaccine. (A) Cartoon depicting the O-antigen ligase (WaaL)-dependent addition of the N-glycan structure to the LPS core of E. coli. (B) Western blot of proteinase K digested whole cell lysates of E. coli wzy::kan (pACYC184 (pglmut)), lane 1 and E. coli wzy::kan (pACYC184), lane 2 probed with R1-4 are shown. The formation of the LPS-core-N-glycan molecule is indicated by an arrow. Molecular weight markers (in kDa) are indicated on the left. (C) FACS analysis of 2 × 10⁴ cells of E. coli wzy::kan (pACYC184 (pglmut)) in light grey and E. coli wzy::kan (pACYC184) in dark grey. Fluorescent intensity is shown on the x-axis, cell counts (arbitrary units) are shown on the y-axis. (D) The sequence of the LPS core N-glycan fusion product is shown. N-glycan-derived monosaccharide residues that could be assigned by NMR are shown. Capital letters refer to residues as outlined in Supplementary Table 2. (E) NMR spectrum of the purified LPS core-C. jejuni-N-glycan compound. Correlations from anomeromic protons (as indicated) are shown. Green, COSY; red, TOCSY; black, ROESY.
components gave transglycosidic Nuclear Overhauser effects of 1:4 and 1:6, all signals of the outer LPS-core l-glycero-d-manno-heptose (Hep, L in Table S2) were found by the analysis of the main heap of correlations and the N-glycan was linked to the O-7 of Hep (Fig. 2D). However, instead of diNAcBac that constitutes the native C. jejuni N-glycan reducing end sugar, GlcNAc was found at this position which has previously been observed when expressing the C. jejuni N-glycan structure in E. coli.

N-glycan based vaccines reduce C. jejuni colonization in chickens. In a 35-day SPF Leghorn chicken challenge model, we tested the efficacy of each vaccine composition as well as the best dosage and route of administration. The glycoprotein vaccines were injected into the breast or in the leg while whole cell vaccines (live or inactivated) were orally gavaged. First, we determined the C. jejuni challenge and the protein glycoconjugate doses (Fig. 3A). Birds that were challenged with $1 \times 10^2$ and $1 \times 10^6$ C. jejuni cells on day 28 showed comparable colonization levels. Vaccination in the breast on days 7 and 21 with 5 \(\mu\)g or 100 \(\mu\)g of purified glycosylated ToxC-GT protein resulted in a statistically significant reduction in bacterial colonization (p-values < 0.05).
No detectable N-glycan-specific antibody titres were present in non-vaccinated birds, in birds that received the inactivated strain (p-value < 0.05). Similarly, vaccination with non-glycosylated ToxC-GT led to similar colonization levels when compared to the positive control group (Fig. 3A). No C. jejuni above the detection limit of 1 × 10^2 CFU/gram cecal content was observed in negative control birds. Next we tested if the injection site influences the efficacy of the protein vaccine (Fig. 3B). The colonization level after challenge with 1 × 10^2 C. jejuni dropped significantly from 2 × 10^9 to 2 × 10^6 (p-value < 0.005) and 9 × 10^2 (p-value < 0.05) colony forming units (CFU) per gram cecal content in birds vaccinated in the chest or in the leg on days 7 and 21. Levels of C. jejuni in negative control birds were below the detection limit. In comparison, we also tested the efficacy of inactivated E. coli cells displaying the N-glycan that were administered by oral gavage. For this treatment, a statistically significant drop (p-value < 0.005) in the C. jejuni load after challenge to 3 × 10^6 CFU per gram cecal content was observed (Fig. 3B). Subsequently, we tested the live E. coli vaccine. In two independent studies, birds were orally vaccinated with the E. coli strain expressing the LPS core-N-glycan on its surface. C. jejuni colonization after challenge was significantly reduced (p-value < 0.005) when compared to colonization levels in unvaccinated birds (Fig. 3C,D). In contrast, no statistically significant difference (p-value > 0.05) in the colonization levels was observed in birds that received the isogenic E. coli strain not expressing the C. jejuni N-glycan structure on its surface (Fig. 3D).

We also determined the relative levels of E. coli in birds that received the live vaccine. No E. coli was detected prior to the first E. coli gavage (day 7). The levels of E. coli declined when monitored at 2, 5 and 9 days after the first vaccination (Supplementary Table 3 and Supplementary Methods). A more rapid reduction of E. coli was observed after the second gavage (day 21) with no detectable E. coli was observed after the second gavage (day 21) with no detectable E. coli in birds that were given the control strain. No E. coli was detected on day 35 clearly showing that the vaccine strain is self-limiting. Interestingly, the live E. coli vaccine expressing the N-glycan appeared to be cleared faster when compared to the E. coli control strain.

**Vaccinated birds develop an N-glycan specific IgY response.** N-glycan-specific immune responses were determined in sera taken at day 28 prior to C. jejuni challenge (Fig. 3E). The average immune response against each vaccine corresponded to the degree of protection against C. jejuni colonization; however, the highest individual titres did not correlate with birds showing the lowest levels of C. jejuni colonization. The highest titres with a statistically significant increase in the IgY levels when compared to negative control birds, were observed in birds vaccinated with the live E. coli strain expressing the N-glycan (p-value < 0.005) on its surface followed by birds that received the inactivated strain (p-value < 0.05). However, the increase in the median between these two groups was not statistically significant (p-value > 0.1). Although some birds that received the protein glycoconjugates also showed an increase in the N-glycan-specific IgY levels when compared to negative control birds, the increase in the median did not result in an overall statistically significant increase (p-values > 0.05). No detectable N-glycan-specific antibody titres were present in non-vaccinated birds, in birds that received the E. coli strain not expressing the N-glycan, and in birds that received non-glycosylated ToxC-GT-His_6 (not shown) indicating that the observed increase in IgY titres was due to the presence of the C. jejuni N-glycan on either the surface of E. coli or when N-linked to the ToxC-GT Hs₆ protein.

**Vaccination of chickens does not select for C. jejuni resistant strains.** Although some birds vaccinated with the live E. coli N-glycan expressing strain showed non-detectable levels of C. jejuni colonization, others still had low levels of colonization (Fig. 3C,D). Those isolated C. jejuni colonies were probed with the N-glycan-specific R1-4 antiserum (Supplementary Fig. 1). Every colony showed strong reactivity with the antiserum verifying that these isolates still express the N-glycan and that no selection for N-glycan-specific R1-4 antiserum (Supplementary Fig. 1). Every colony showed strong reactivity with the antiserum verifying that these isolates still express the N-glycan and that no selection for N-glycan-resistant strains.

**Vaccination prevents C. jejuni colonization without changes in the resident bacterial community in the Leghorn chicken intestine.** In birds from the positive control groups that were initially inoculated with 1 × 10^6 CFU of C. jejuni and subsequently showed colonization levels up to 1 × 10^9 to 10^10 CFU/gram cecal content, the presence of C. jejuni caused a shift in the global structure of the resident bacterial communities, as shown in a non-metric multidimensional scaling (NMDS) ordination plot based on Bray Curtis metrics (Fig. 4A) and PCoA on weighted UniFrac metrics (data not shown). Vaccination with the live E. coli-based vaccine reversed these changes, causing the gut microbiota of vaccinated birds to shift back to the composition observed in the negative control group that did not receive C. jejuni. Assessment of alpha diversity (within samples) showed no significant differences between these two treatment groups (Fig. 4B). Inoculation with C. jejuni led to major colonization by the species, leading to a significant increase in the relative abundance of microbes from the phylum Proteobacteria (increased from 12% to 35%), the family Campylobacteraceae (increased from less than 0.001 to 19%), the genus Campylobacter (increased from less than 0.001% to 19%) and the species (OTU) C. jejuni/ C. subtilarcticus (increased from less than 0.001% to 22%) (Fig. 4C) demonstrating that C. jejuni establishes itself in the chicken gut without decreasing diversity or changing the resident community, supporting its non-pathogenic status. Vaccination led to a substantial reduction in colonization of C. jejuni (less than 0.001%, p < 0.05), supporting our culture-based findings. Few other significant changes in the microbiota were detected. An OTU related to Clostridium tertium decreased from 20% in the negative group, to 11% in the positive, and further reduced to 6% in the vaccinated group (p = 0.0444) (Fig. 4C).
Discussion

Three major strategies for reducing C. jejuni infection in poultry have been identified: (1) reduction of environmental exposure (e.g. biosecurity measures), (2) measurements to decrease C. jejuni in the chicken gut (e.g. vaccination), and (3) the use of antimicrobial alternatives (e.g. bacteriophage therapy or bacteriocin treatment). Except for biosecurity measures, these approaches are still under development. Active immunization of poultry would be an attractive alternative to administering antibiotics to decrease the abundance of C. jejuni in the native host and the resulting diarrheal disease in humans.

An effective vaccine against C. jejuni in poultry has to meet three main challenges: (1) the identification of cross-protective antigens, (2) the induction of a rapid and strong immune response, and (3) the development of novel adjuvants to further stimulate immunity against C. jejuni. The C. jejuni N-glycan fulfills all of these requirements. It is surface exposed, immunogenic in humans and rabbits and, as demonstrated in this study, induces a protective immune response in chickens. In addition, lipopolysaccharide present in the live E. coli vaccine as well as the use of the toxoid, ToxC, in the ToxC-GT-C. jejuni N-glycan-His, glycoconjugate act as natural adjuvants to stimulate the immune system. Since the N-glycan is the only glycoconjugate structure conserved among all C. jejuni isolates, we would expect the N-glycan specific immune response to be cross-protective.

Both, our recombinant glycoprotein (GlycoTag) and whole cell delivery approaches result in a multivalent presentation of the N-glycan. Multivalent presentation of group B streptococcus carbohydrate epitopes was demonstrated to be significantly more efficient than currently available vaccines that have a lower carbohydrate to protein ratio. Similarly, a vaccine with two to five CPS per CRM197 was sufficient to induce a protective immune response in mice and monkeys against challenge with C. jejuni 81–176. Although we observed a reduction in C. jejuni colonization with the administration of higher doses of the glycoconjugate vaccine after challenge with 10^2 as well as 10^6 C. jejuni CFU, the lower challenge dose is probably more reflective of the natural conditions when C. jejuni is first introduced into the flock, e.g. through flies that enter the poultry houses. Artificial fly feeding studies demonstrated that C. jejuni levels are not higher than 1 × 10^4 CFU and it has been shown that...
as low as 40 colony forming units of *C. jejuni* are sufficient to induce chicken colonization, however, the infectious dose varies between strains of *C. jejuni*.25,29

The presentation of the N-glycan on the *E. coli* cell surface is possible due to the interplay between the endogenous O-antigen LPS and the heterologous N-glycan biosynthesis pathways60–62 and their requirement for undecaprenylphosphate for sugar assembly. Interestingly, WecA, the initiating GlcNAc transferase involved in enterobacterial common antigen and O-antigen biosynthesis can substitute for *C. jejuni* PglC function however, preferring UDP-GlcNAc rather than UDP-diNAcBac as the initiating monosaccharide61. Although pglC (on pACYC184pgl 

N-glycan itself is required for chicken colonization and protects the cell from proteolytic attack by chicken gut enzymes. If N-glycan was present on the cell surface of the *E. coli* expressing the N-glycan on their surface significantly reduced colonization. The overall levels of IgY antibodies were in agreement with the level of protection after challenge indicating that the response is protective.

The use of live *E. coli* as a self-limiting carrier for the N-glycan antigen is favorable over previously used Salmonella-based delivery systems that might prove difficult to introduce with respect to food standards in certain countries. In addition, the *E. coli* vaccine is easy to produce and to administer compared to the use of individual protein antigens that have to be purified large-scale and potentially administered by subcutaneous injections to reach their full potential. These facts will allow the testing of the vaccine in large scale applications using other popular breeds of chickens for the creation of a low cost vaccine for *C. jejuni* reduction.

**Methods**

**Bacterial strains, plasmids and growth conditions.** *C. jejuni* strain 81–176 was grown on Mueller Hinton (MH) agar (Difco) at 37 °C under microaerobic conditions (85% N2, 10% CO2, and 5% O2). *Escherichia coli* strains were grown on Luria Bertani (LB) or 2-times YT (2xYT) medium supplemented with ampicillin (Amp), kanamycin (Km), or chloramphenicol (Cm) at a final concentration of 100, 50 or 20 μg/ml where needed. Karmali supplement (if required) was added according to the instructions of the manufacturer (Oxoid). Bacterial strains and plasmids are summarized in Supplementary Table 1.

**Bioinformatic analyses of the C. jejuni proteome.** FASTA protein sequences from *C. jejuni* species available from the EMBL server (http://www.ebi.ac.uk/) were used to perform an amino acid motif search using the protein pattern find software Sequence Manipulation Suite: Protein Pattern Find (http://bioinformatics.org/sm22/protein_pattern.html, with (d|e).n.(s|t) as the search criteria that matches the requirement for the bacterial N-linked glycosylation site D/E-X1-N-X2-S/T. Since positions X1 and X2 do not tolerate a proline48, only GlcNAc was found to be the linking sugar to the O-7 of the L-glycerol-3-manno-heptose of the LPS-core. One explanation might be that although the *E. coli* K-12 Waal A-antigen ligase has been reported to lack substrate specificity63, GlcNAc containing N-glycan LLOs are preferred over diNAcBac containing LLOs. One might argue that the absence of diNAcBac could negatively influence the protective immune response against the *C. jejuni* N-glycan, but we have previously demonstrated that the immune response against the N-glycan is targeted against the non-reducing end residues35,64–66.

We demonstrated that the N-glycan was still present on all *C. jejuni* isolates after passage through vaccinated birds indicating there was no selection for N-glycan-negative *C. jejuni* variants. This is not surprising since the pgl genes are lacking homopolymeric tracts that are subject to high-frequency slip-strand mutation as shown for genes encoding *C. jejuni* O-linked glycans, LOS and CPS structures67–71. In the unlikely event that selection against the N-glycan would occur, these cells would not be able to survive in the chicken gut since the N-glycan itself is required for chicken colonization and protects the cell from proteolytic attack by chicken gut proteases35,42–44.

The use of the conserved N-glycan structure in vaccine compositions significantly reduces *C. jejuni* at the source. We show that treatment with protein-based glycoconjugates significantly reduces *C. jejuni* colonization after challenge with the organism independent of the injection time point or the application site. Oral vaccination with live *E. coli* cells expressing the N-glycan on their surface significantly reduced colonization. The overall levels of IgY antibodies were in agreement with the level of protection after challenge indicating that the response is protective.

The use of live *E. coli* as a self-limiting carrier for the N-glycan antigen is favorable over previously used Salmonella-based delivery systems that might prove difficult to introduce with respect to food standards in certain countries. In addition, the *E. coli* vaccine is easy to produce and to administer compared to the use of individual protein antigens that have to be purified large-scale and potentially administered by subcutaneous injections to reach their full potential. These facts will allow the testing of the vaccine in large scale applications using other popular breeds of chickens for the creation of a low cost vaccine for *C. jejuni* reduction.

**Cloning, expression and validation of the glycosylated GlycoTag fusion protein.** The gene encoding an enzymatically inactive and nontoxic form of the diphtheria toxin (toxoid, *toxC*) from *Corynebacterium diphtheriae* was amplified from plasmid pPDT127 with oligonucleotides CS-378 (′ 5′- ATATATATATGCCTCCTGATGATGCTTTGATTTC3′) and CS-379 (′ 5′- ATATACTCGAGTGTTTGTGATGCTTTGATTTC3′) to introduce NcoI and XhoI sites, respectively. The obtained NcoI-XhoI digested PCR product was inserted into plasmid pET22b cut with the same enzymes translocationally fusing the gene to the plasmid-derived pelB secretion sequence for the transport of the product into the periplasmic space. A 271 bp DNA fragment including the 9 N-glycosylation sequon repeat (GlycoTag, GT) was amplified from chromosomal DNA of *C. jejuni* 11168 with oligonucleotides CS-334...
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in PBS supplemented with an EDTA free protease inhibitor cocktail according to the instructions of the manufacturer. Cells were spun out of the mixture and the supernatant was used to repeat the procedure 4X. The resulting serum (R1-4) that was depleted of E. coli was resuspended in PBS that corresponded to 1/3 of the original volume of the OD600.

Preparation of E. coli cells for downstream processing. E. coli K12 wzy:kan (KEIO collection) was transformed with plasmids pACYC184 (pglmut) and pACYC184. Whole cells for vaccination and verification of antigen expression were prepared as follows: overnight cultures were used to inoculate fresh 2xYT broth to an OD600 of 0.1. Cells were grown at 37 °C until an OD600 of at least 1.0 was reached. Cells were cooled on ice for 15 min, protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and cells were grown for an additional 18 hrs at 30 °C. Cells were cooled on ice, harvested by centrifugation (15 min, 4,200 × g, 4 °C), and resuspended in PBS supplemented with an EDTA free protease inhibitor cocktail according to the instructions of the manufacturer (Roche). Cells were disrupted in a cell disrupter (Constant Systems, Ltd.), the resulting suspension was centrifuged for 30 min at 13,000 × g, 4 °C, and the resulting supernatant was loaded onto a 1 ml Ni-NTA column using the AAKTA purification system (GE Healthcare). After an initial wash step with 10 mM imidazole in PBS, an imidazole gradient was applied from 10–250 mM over 50 column volumes. Elution fractions that contained the ToxC-GT-His6 protein were analyzed by 12.5% SDS-PAGE, combined and the glycosylation status of ToxC-GT-His6 was verified by Western blotting as described previously.

Preparation of protein free cell extracts. Briefly, E. coli cells of a culture equivalent to an OD600 of 1.0 (prepared as described above) were centrifuged, resuspended in 100 μl of 1 × Laemml sample buffer, and heated to 95 °C for 10 min. Proteinase K (Fermentas) was added to a final concentration of 200 μg/ml and the sample was incubated at 60 °C for 1 h. Glycolipid species from the proteinase K-digested whole cell lysates were separated by 12.5% SDS-PAGE, transferred to PVDF membranes and analyzed by Western blotting as described previously.

Cross absorption of R1 antisera. C. jejuni N-glycan-specific antisera (R1) was cross absorbed using whole cells of E. coli wzy:kan (pACYC184) as follows: the pellet of 1 ml of OD600 = 1.0 culture was blocked with 1 ml PBS and 5% skim milk for 20 min. Cells were spun for 5 min at 4,200 × g, resuspended and incubated with 1 ml of R1 serum for 30 min on ice with occasional inversion of the tube. Cells were spun out of the mixture and the supernatant was used to repeat the procedure 4X. The resulting serum (R1-4) that was depleted of E. coli-specific antibodies was used for downstream analyses.

Western blotting. Western blots were performed as previously described. C. jejuni N-glycan-specific antisera, R1, or cross-absorbed R1 (R1-4) was used at a 1:7,500 dilution, anti–His antisera (Santa Cruz Biotech) was used at a 1:1,000 dilution, and AP-conjugated rabbit and mouse antisera (Santa Cruz Biotech) were used at 1:2,000 dilutions. Immunoreactive bands were visualized directly on the PVDF membrane using the NBT-BCIP detection reagents (Promega) according to the instructions of the manufacturer.

Colony lifts. Cecal content dilutions were plated on MH agar supplemented with the Karmali supplement and Trimethoprim. Colony lifts were performed as previously described. Immunodetection was done as for Western blotting (described above).

Fluorescence activated cell sorting (FACS). E. coli cells were adjusted to OD600 of 1.0 and 1 ml was pelleted by centrifugation and resuspended in 1 ml blocking solution (PBS, 5% skim milk). Cells were probed
with R1-4 and Alexa Flour-546 conjugated anti-rabbit antiserum, and analyzed by FACS (on a LSR-Fortessa Flow Cytometer). FACS data were processed with the FACS Diva software. DAPI counter-staining was used to identify and gate for intact cells.

NMR. Glycolipids were prepared from cell pellets obtained from eight litres of \( \text{OD}_{600} = 1.0 \) *E. coli* wzy:kan (pACYC184p::pgl) cells grown as described above. LPS was extracted by phenol-water, dialyzed, treated with acetic acid (AcOH) to precipitate nucleic acids, dialyzed, dried, hydrolyzed with 2% AcOH and separated on Biogel P6. Fractions were analyzed by NMR. Fractions that contained *C. jejuni* N-glycan signals were combined and separated on an anion-exchange Hitrap column using an NaCl gradient. Fractions were again analyzed by NMR. *C. jejuni* N-glycan LPS-core components eluted as a broad peak after the enterobacterial common antigen peak (data not shown). Fractions containing *C. jejuni* N-glycan signals were desalted by Sephadex G-15 chromatography. Connections were confirmed by NOE and HMBC.

**Chicken challenge studies.** Animal studies were carried out in accordance with the protocol approved by the Animal Care and Use Committee at the University of Alberta using a 35 day challenge protocol. In general each group contained up to 8 birds (SPF Leghorns, Poultry Research Facility, University of Alberta) that were randomly tested for the presence of *C. jejuni* on the day of hatch (day 1) by plating cloacal swabs onto selective Karmali agar. In all cases no *C. jejuni* colonies were observed on plates after 48 hr of incubation under microaerobic conditions at 37°C.

**Chicken vaccination.** To test the efficacy of the protein glycoconjugates, birds received 300 µl of protein antigen complete adjuvant for the 1st vaccination (day 7) and the same amount but with Freund’s incomplete adjuvant for the 2nd vaccination (day 21). Antigens were injected at two sites in the chest with 150 µl of vaccine formulation per site or in the leg with 150 µl of vaccine formulation in each leg. Control groups received PBS in Freund’s complete/incomplete instead of protein. Vaccination with whole cells of *E. coli* was done by orally gavaging 300 µl of PBS containing 1 × 10^8 live or inactivated (cross-linked, as described above) *E. coli* cells on days 7 and 21. Control groups were gavaged with 300 µl of PBS only. In the case of the *E. coli* whole cell live vaccine, cloacal swabs taken at various time points were plated onto LB Km-Cm. Relative CFUs for each bird were determined by colony counts after 18 hr of incubation at 37°C.

**Campylobacter challenge.** Birds were orally gavaged (challenged) on day 28 with either PBS (negative control) or with 300 µl PBS containing 10^5 or 10^6 *C. jejuni* strain 81–176 cells. To prepare the challenge, *C. jejuni* 81–176 was grown for 18 h on MH agar and harvested with cold MH broth. Cells were washed twice with cold PBS and adjusted to an OD_{600} of 1.0 (OD_{600} of 1.0 equals 1.5 × 10^9 cell/ml). Serial dilutions in PBS were performed dependent on the final amount of cells that were administered. For example: 3 × 10^2 cells/ml (=1 × 10^2 cells per 300 µl = 1 dose). Cells were maintained on ice until used. Birds were maintained for 7 days after challenge and then euthanized according to the approved guidelines of the Canadian Council for Animal Care. Ceca were collected, the contents were removed and weighed and adjusted to 1 mg cecal content per 1 ml with sterile PBS. Aliquots of 10-fold serial dilutions (in PBS) of the cecal contents were plated on selective Karmali agar. CFUs were determined after incubation of the plates for 48 hr under microaerobic conditions.

**Serum preparation.** Blood samples were collected on day 7 (50 µl pre-bleed) and day 28 (100 µl vaccine response prior to challenge). Fresh blood samples were kept at room temperature for at least 18 hr or at 37°C for at least 6–8 hr, until a firm blood clot was formed. Samples were centrifuged (5 min, 13,000 × g, 4°C) and the supernatant (serum) was transferred to a fresh tube. After addition of glycerol to a final concentration of 10%, the sera were stored at −20°C until further use.

**ELISA testing for N-glycan-specific antibodies.** We developed a 96-well plate ELISA assay to quantify the N-glycan-specific IgY responses in vaccinated birds. fOS from *C. jejuni* (Cj) was prepared as described and coupled to BSA by reductive amination as previously described. Formation of the BSA-Cj-N-glycan conjugate was confirmed by Western blotting using R1-4 as a primary antibody. After adjusting the concentration to 1 mg/ml using PBS, the glycoconjugate was stored at 4°C until further use. We first tested the BSA-Cj-N-glycan conjugate binding capacity by coupling increasing amounts of the antigen and probed with R1-4 antiserum. A linear increase in signal intensity was observed when higher concentrations of the BSA-Cj-N-glycan conjugate were added to each well, therefore 500 ng of BSA-Cj-N-glycan conjugate per well were used for further analyses.

Then, 96-well Maxisorp plates (Thermo Fisher) were coated with 500 ng of BSA-Cj-N-glycan conjugate overnight (18 hr) at 4°C. After removal of unbound antigen, the plate was blocked for 1 hr at RT with 5% skim milk in 100 µl PBS-T with shaking. After discarding the blocking solution, 100 µl of the antibody solution was added and incubated for 1 hr as described above. Antibody solutions were R1-4 antiserum diluted 1:1000 in PBS-T with 1% skim milk, or chicken serum (prepared from day 28 bleeds from the 2nd vaccination experiment, Fig. 3B), diluted 1:10 in PBS-T with 1% skim milk. Plates were incubated for 1 hr at RT as described and each well was washed 3 times for 5 min with 100 µl of PBS-T. After addition of 100 µl of the secondary antibody solution (either anti-rabbit-AP (1:500), for the R1-4 control or anti-chicken IgY (1:500) for the experimental samples and incubated for 1 hr at RT), the secondary antibody solutions were discarded and the wells were washed 4 × 5 min with 100 µl of PBS-T. After the last washing step, the remaining washing solution was completely removed from each well and the plates were developed using pNPP as a substrate following the instructions of the manufacturer (Thermo Fisher). Immunoreactivity in each serum was determined after scanning the plate at OD 405 in a plate reader.
Chicken microbiome studies. Chicken microbiome studies were performed to analyze and compare the composition of the bacterial community in negative control (non-vaccinated, not challenged) and positive control (non-vaccinated, challenged) birds in comparison with birds that were challenged with *C. jejuni* after they received the *E. coli* live vaccine strain that expresses the N-glycan on its surface.

DNA isolation. First, 250–300 mg of cecal contents were placed in a 2 ml tube and washed with cold 1xSTE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 8.0). The sample was spun at low speed (1,000 rpm) to remove large pieces of unwanted debris. The supernatant was placed in a new 2 ml tube and spun at a higher speed (14,500 rpm on the mini-spin) to pellet the bacteria in the sample. The supernatant was removed; the pellet was resuspended by vortexing and was washed twice with 1 ml of ice cold 1xSTE buffer. After removing the supernatant, 180 μl of Qiagen ATL was added with 20 ml of Roche PK and digested overnight at 56 °C on a rotisserie. The DNA was extracted on the Biosprint using the Biosprint_96 DNA tissue and blood kit according to the QIAGEN protocol. The DNA samples were quantified using the Promega QuantiFlour® dsDNA System kit.

Library preparation and quantification. Extracted DNA from chicken cecum samples was initially amplified using the universal primers 926F (5′-AAACTYAAAKGAATWGRCGG-3′) and 1392R (5′-ACGCGGCTTGTWGGTRC-3′) that targets the V6 to V8 region of the 16S ribosomal RNA gene with PCR conditions as detailed in the 16S Metagenomic Sequencing Library Preparation (Illumina®, San Diego, CA). A bioanalyzer trace of amplified products was obtained using the DNA 1000 chip on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Amplicons with a single product at approximately 500 bp were determined to be suitable for further library preparation. Subsequently PCR cleanup was carried out using the Agencourt Ampure XP beads (Beckman Coulter, Mississauga, ON). Nextera XT Dual indexing barcodes adapters (Illumina®) were attached to the bead-cleaned amplicons by a second PCR as detailed in the 16S Metagenomic Sequencing Library Preparation (Illumina®). Barcoded amplicons were cleaned up by an additional step of Agencourt Ampure XP bead clean up (Beckman Coulter). ABI Veriti 96 well Thermal Cycler (Life Technologies, Burlington, ON) was used to run all the PCR reactions. Library quality was assessed by running the DNA1000 chip on the Agilent 2100 Bioanalyzer (Agilent Technologies). Library sizes ranged from 630 to 670 bp. Qubit HS dsDNA Assay (Life Technologies) was used to quantify the libraries. Individual libraries with their respective barcodes were pooled in a 4 nM library pool. The 16S rRNA gene sequencing was carried out on the Illumina MiSeq (Illumina) generating 300 bp reads in both the forward and reverse directions.

Microbiome - data analysis. Illumina 16S RNA sequence reads were processed and analyzed as previously described with minor modifications as follows. Reads were trimmed to 300 bp using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and paired-end reads were merged using the merge-illumina-pairs application (https://github.com/merem/illumina-utils) with the following quality parameters: p-value = 0.03, enforce Q30 check, no ambiguous nucleotides and perfect matching to primers. An average of 227,278 merged reads per sample was obtained. Files exceeding 150,000 reads were subsampled to that amount of reads using MOTHUR v.1.32.0 to standardize the depth of analysis across samples, while all reads were kept for two samples in the dataset that had less than 150,000 reads (84,462 and 101,732 reads). Merged sequences between 440 and 470 bp long were kept for analysis. USEARCH v.7.0.1001 was used to remove potential chimeras and to cluster the reads into operational taxonomic units (OTUs) using a 98% similarity cut-off. Taxonomic classification for the OTUs was done by selecting the highest percent identity for the OTUs representative sequences (selected by the UPARSE-OTU algorithm based on read abundance) when blasted against the Greengenes database, and confirmed through NCBI Blast and RDP SeqMatch. Percent proportions were calculated based on the total number of reads per sample. Diversity metrics were calculated using MacQIIME version 1.8.0. One-way analysis of variances (ANOVA) with Tukey’s post hoc test was used to compare bacterial composition and differences in diversity between the treatments. Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

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Author Contributions
H.N., J.W., C.C. and C.M.S. conceived and designed the research; H.N., B.D., Y.Y.L., M.E.P.-M. and E.V. performed the experiments; H.N., Y.Y.L., M.E.P.-M., E.V., J.W., C.C. and C.M.S. analyzed the data; H.N., M.E.P.-M., J.W., C.C. and C.M.S. wrote and revised the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare competing financial interests. Christine Szymanski is one of the founders of VaxAlta Inc, a company dedicated to creating glycoconjugate vaccines for livestock. Christine Szymanski and Harald Nothaft are inventors on a patent describing the C. jejuni vaccine.

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