Erythrocyte and Porcine Intestinal Glycosphingolipids Recognized by F4 Fimbriae of Enterotoxigenic

**Escherichia coli**

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**Abstract**

Enterotoxigenic F4-fimbriated *Escherichia coli* is associated with diarrheal disease in neonatal and postweaning pigs. The F4 fimbriae mediate attachment of the bacteria to the pig intestinal epithelium, enabling an efficient delivery of diarrhea-inducing enterotoxins to the target epithelial cells. There are three variants of F4 fimbriae designated F4ab, F4ac and F4ad, respectively, having different antigenic and adhesive properties. In the present study, the binding of isolated F4ab, F4ac and F4ad fimbriae, and F4ab/ac/ad-fimbriated *E. coli*, to glycosphingolipids from erythrocytes and from porcine small intestinal epithelium was examined, in order to get a comprehensive view of the F4-binding glycosphingolipids involved in F4-mediated hemagglutination and adhesion to the epithelial cells of porcine intestine. Specific interactions between the F4ab, F4ac and F4ad fimbriae and both acid and non-acid glycosphingolipids were obtained, and after isolation of binding-active glycosphingolipids and characterization by mass spectrometry and proton NMR, distinct carbohydrate binding patterns were defined for each fimbrial subtype. Two novel glycosphingolipids were isolated from chicken erythrocytes, and characterized as galactosylceramide, sulfatide (SO3-3Galβ1Cer), sulf-lactosylceramide (SO3-3Galβ4Glcβ1Cer), and globo-tetraosylceramide (Galβ3GalNAcβ3Galβ4Glcβ1Cer). These two compounds, and lactosylceramide (Galβ4Glcβ1Cer) with phytosphingosine and hydroxy fatty acid, were recognized by all three variants of F4 fimbriae. No binding of the F4ad fimbriae or F4ad-fimbriated *E. coli* to the porcine intestinal glycosphingolipids occurred. However, for F4ab and F4ac two distinct binding patterns were observed. The F4ac fimbriae and the F4ac-expressing *E. coli* selectively bound to galactosylceramide (Galβ1Cer) with sphingosine and hydroxy 24:0 fatty acid, while the porcine intestinal glycosphingolipids recognized by F4ab fimbriae and the F4ab-fimbriated bacteria were characterized as galactosylceramide, sulfatide (SO3-3Galβ1Cer), sulf-lactosylceramide (SO3-3Galβ4Glcβ1Cer), and globo-triaosylceramide (Galβ4Galβ4Glcβ1Cer) with phytosphingosine and hydroxy 24:0 fatty acid. Finally, the F4ad fimbriae and the F4ad-fimbriated *E. coli*, but not the F4ab or F4ac subtypes, bound to reference gangliotriaosylceramide (Galβ3GalNAcβ3Galβ4Glcβ1Cer), gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer), isoglobo-tetraosylceramide (Galβ3Galβ4Glcβ1Cer), and neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer).

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**Introduction**

Adhesion of microbes and microbial toxins to their target tissue by binding to cell surface carbohydrates is nowadays textbook knowledge, as e.g. the binding of influenza virus to sialic acid-containing glycoconjugates, cholera toxin to the GM1 ganglioside, and uropathogenic P-fimbriated *Escherichia coli* to Galβ4Gal-containing glycosphingolipids. However, for many microbial adhesins the target cell receptors have not yet been identified. Enterotoxigenic *E. coli* (ETEC) infections are a major cause of diarrhea in young pigs. Infecting bacteria adhere to and colonize the intestinal epithelium and cause diarrhea primarily by the production of heat-labile and/or heat-stable enterotoxin (LT and ST respectively). Adherence is mediated by fimbrial structures, and porcine ETEC primarily express five types of fimbriae designated F4 (K88), F5 (K99), F6 (987P), F41 and F18. F4 fimbriae are the most prevalent fimbrial structures expressed by porcine ETEC causing diarrhea and mortality in newborn, suckling and newly weaned piglets [1]. The F4 fimbriae are composed of a large number of the major subunit FaeG, with a small number of minor subunits interspersed throughout the structure [2]. F4 fimbriae are expressed by the *fae*-operon, coding for the regulatory proteins FaeA and FaeB [3], the fimbrial tip protein FaeG [4], the usher FaeD [5], the chaperone FaeE [6], the minor fimbrial shaft subunits FaeF and FaeH [7], the adhesive major subunit FaeG [8], and the minor subunits FaeI and FaeJ with undefined roles.

There are three antigenically distinct variants of F4 fimbriae designated F4ab, F4ac and F4ad, respectively, distinguished by...
amino acid substitutions in the FaeG subunit [2,9,10]. Studies on the interactions of the variants of F4 fimbriae with erythrocytes, intestinal mucus and intestinal epithelial cells have demonstrated that the F4ab, F4ac and F4ad fimbriae have different, but related, carbohydrate binding specificities (reviewed in [2]), and several receptor candidates for each variant have been suggested. Studies of the hemagglutinating properties of F4ab-, F4ac- and F4ad-expressing E. coli, using a panel of erythrocytes from different species, showed that rabbit and guinea pig erythrocytes were agglutinated in a mannoside-resistant manner by all three serotypes [9]. Pig and rat erythrocytes were agglutinated by F4ab- and F4ad-fimbriated E. coli, while chicken erythrocytes were selectively agglutinated by F4ab-fimbriated bacteria.

Previous studies of the carbohydrate recognition of F4ab-fimbriated E. coli, using reference glycosphingolipids, demonstrated a binding to galactosylceramide (Galβ1Cer) with hydroxy ceramide, lactosylceramide (Galβ1Glcβ1Cer), and gangliotetraosylceramide (GalαNeββ3Galβ4Glcβ1Cer), and gangliotetraosylceramide (Galβ3Galβ4Glcβ1Cer), leading to the conclusion that a Galβ1 was necessary for binding to occur [11]. Another glycosphingolipid binding study demonstrated binding of F4ab-, F4ac- and F4ad-fimbriae to lactotriosylceramide (GalαNeββ3Galβ4Glcβ1Cer), neolactotetraosylceramide (Galβ4Glcβ1Cer), and neo-lactohexaosylceramide (Galβ4Glcβ1Cer), leading to the conclusion that a Galβ1 was necessary for binding to occur [11]. One other glycosphingolipid binding study demonstrated binding of F4ab-, F4ac- and F4ad-fimbriae to lactotriosylceramide (GalαNeββ3Galβ4Glcβ1Cer), neolactotetraosylceramide (Galβ4Glcβ1Cer), and neo-lactohexaosylceramide (Galβ4Glcβ1Cer), leading to the conclusion that a Galβ1 was necessary for binding to occur [11].

In order to get a comprehensive view of the F4-binding glycosphingolipids involved in F4-mediated hemagglutination and adhesion to the epithelial cells of porcine intestine, the binding of isolated F4 fimbriae, and F4ab-fimbriated E. coli, to glycosphingolipids from erythrocytes (human, chicken, guinea pig, rabbit and porcine) and from porcine small intestinal epithelium was examined in the present study. Specific interactions between the F4ab, F4ac and F4ad fimbriae and both acid and non-acid glycosphingolipids were obtained, and after isolation of binding-active glycosphingolipids and characterization by mass spectrometry and proton NMR, distinct binding patterns were defined for each fimbrial subtype.

**Results**

Characterization of native and mutant F4 fimbriae

Wild type and deletion mutant F4 fimbriae were isolated for glycosphingolipid binding experiments. When analyzed by SDS-PAGE the isolated F4 fimbriae variants all migrated as single bands, which represent the major subunit FaeG, and the apparent molecular weight of the proteins was in agreement with the predicted molecular masses (Figure S1) [2].

Binding of F4 fimbriae and F4-fimbriated E. coli to erythrocyte glycosphingolipids

The initial F4 binding studies were done using mixtures of total acid and non-acid glycosphingolipids isolated from human, chicken, guinea pig, rabbit and pig erythrocytes. No binding to the acid glycosphingolipid fractions was obtained (data not shown). However, all three fimbriae, and the three corresponding variants of F4-fimbriated bacteria, selectively bound to three compounds in the non-acid glycosphingolipid fraction of chicken erythrocytes (Fig. 1, lane 2). The binding-active compounds migrated in the mono-, tetra- and hexaglycosylceramide regions, respectively. In addition, the F4ad fimbriae and the F4-fimbriated E. coli distinctly bound to the major compound of guinea pig erythrocytes, migrating in the triglycosylceramide region (Fig. 1D and G, respectively, lane 3). This compound was not recognized by the F4ab or F4ac fimbriae or the bacteria expressing F4ab or F4ac fimbriae. The major glycosphingolipid of guinea pig erythrocytes is gangliotriaosylceramide (Galβ3Galβ4Glcβ1Cer) [14], and gangliotetraosylceramide isolated from this source was recognized by F4ad fimbriae and F4ad-expressing E. coli (see below).

Isolation of the F4 fimbriae binding slow-migrating glycosphingolipids of chicken erythrocytes

Previously characterized glycosphingolipids of chicken erythrocytes are galactosylceramide, lactosylceramide, and the Forssman pentaglycosylceramide (GalβNαββ3Galβ4Glcβ1Cer) [15]. Here we focused on the F4 binding glycosphingolipids of chicken erythrocytes migrating in the tetra- and hexaglycosylceramide regions. These F4-binding glycosphingolipids were isolated by chromatography on an Iatrobeads column, and the fractions obtained were tested for F4ab and F4ad binding activity using 125I-labeled fimbriae (exemplified for F4ad in Fig. 2B). After pooling of binding-active fractions, 0.5 mg of a fraction containing the binding-active compound migrating in the tetraglycosylceramide region (designated fraction C: tetra-I, Fig. 2, lane 2), and less than 100 µg of the binding-active hexaglycosylceramide (designated fraction C: hexa; Fig. 2, lane 5), were obtained. Proton NMR showed that fraction C: tetra-I was a mixture of four glycosphingolipids. This fraction was therefore further separated on an Iatrobeads column and, after pooling of the F4-binding fractions, 0.2 mg was obtained (designated fraction C: tetra-II).

**ESI/MS of fraction C: tetra-II**

ESI/MS of the native fraction C: tetra-II gave a series of pseudomolecular ions [M-H]− at m/z 1282–1394, indicating a tetraglycosylceramide with two HexNac and two Hex, and with sphingosine and hydroxy 16:0-24:0 fatty acids (data not shown). MS2 of the predominant [M-H]− ion at m/z 1282 gave a series of Y and Z ions identifying a glycosphingolipid with HexNac-HexNac-Hex-Hex sequence and with sphingosine and hydroxy 16:0 fatty acid (Figure S2).

Capillary-LC/MS and MS/MS

The oligosaccharides obtained from fraction C: tetra-II by hydrolysis with Rhodococcus endoglucocerebrosidase II, were analyzed by LC-ESI/MS using a graphitized carbon column [16]. The major saccharide of this fraction was detected as a [M-H]− ion at m/z 747, eluting at 21.5–22.4 min (Figure S3A). MS2 of the [M-H]− ion at m/z 747 resulted in a series of prominent C-type fragment ions (C1 at m/z 220, C2 at m/z 423, and C3 at m/z 585) identifying a tetrasccharide with HexNac-HexNac-Hex-Hex sequence (Figure 3B). The 0,2A4 ion at m/z 687 and the 0,2A4-2H2O ion at m/z 669 were obtained by cross-ring cleavages of the 4-substituted Glc of the internal lactose (Galβ1Cer) part. However, no other cross-ring cleavage ions were observed, suggesting that the two terminal HexNacαcs were 3-linked [16].

The major saccharide derived from fraction C: hexa gave a [M-H]− ion at m/z 1112, eluting at 23.0–23.2 min (Fig. 3C). The MS2 spectrum had a series of C-type fragment ions (C2 at m/z 423, C3 at m/z 585, C4 at m/z 787 and C5 at m/z 950) demonstrating a
hexasaccharide with HexNAc-HexNAc-Hex-HexNAc-Hex-Hex sequence (Fig. 3D). The prominent cross-ring \( \text{m/z} \ 687 \), and the accompanying \( \text{m/z} \ 669 \), indicated a 4-substitution of the internal HexNAc, i.e. a type 2 core [16]. Again, the absence of cross-ring cleavage ions suggested that the two terminal HexNAcs were 3-linked.

Proton NMR spectroscopy of fractions Ctetra-I, Ctetra-II and Chexa

The anomeric region of the 600 MHz spectrum of fraction Ctetra-I is shown as two partial spectra on top of the corresponding COSY sections in Fig. 4, and in Fig. 5 the low field portion of the anomeric region (A; Ctetra-I) is compared to the corresponding section of the same fraction having been further purified (B; Ctetra-II). From Figs. 4 and 5, and the spectrum of the following fraction Cpenta (not shown), which almost exclusively turned out to contain the Forssman pentaglycosylceramide (GalNAc(3Galα(4Galß4Glcß1Cer)) [17], it is concluded that the spectrum in Fig. 5B represents two different species, one of which is the Forssman pentaglycosylceramide (labeled D), and a second one representing a novel four-sugar compound (labeled B) to be characterized below. It can be further concluded that the Ctetra-I fraction shown in Figs. 4 and 5A contains two additional minor species besides compounds B and D (labeled A and B).

Figure 1. Binding of F4 fimbriae and F4-fimbriated Escherichia coli to erythrocyte non-acid glycosphingolipid mixtures. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of \( ^{125}\text{I} \)-labeled F4ab fimbriae (B), F4ac fimbriae (C), F4ad fimbriae (D), and \( ^{33}\text{S} \)-labeled F4ab-expressing E. coli (E), F4ac-expressing E. coli (F), and F4ad-expressing E. coli (G). The lanes were: Lane 1, non-acid glycosphingolipids of human erythrocytes blood group A8, 80 \( \mu \text{g} \); Lane 2, non-acid glycosphingolipids of chicken erythrocytes, 40 \( \mu \text{g} \); Lane 3, non-acid glycosphingolipids of guinea pig erythrocytes, 40 \( \mu \text{g} \); Lane 4, non-acid glycosphingolipids of rabbit erythrocytes, 40 \( \mu \text{g} \); Lane 5, non-acid glycosphingolipids of porcine erythrocytes, 40 \( \mu \text{g} \); Lane 6, reference globotetraosylceramide (GalNAc(3Galα(4Galß4Glcß1Cer)) of human erythrocytes, 4 \( \mu \text{g} \). The major compounds visualized with anisaldehyde in (A) are marked with Roman numbers, and the corresponding glycosphingolipid structures are given to the right of the chromatogram.

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Figure 2. Binding of F4ad fimbriae to slow-migrating non-acid glycosphingolipid fractions isolated from chicken erythrocytes. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of \( ^{125}\text{I} \)-labeled F4ad fimbriae (B). The lanes were: Lane 1, non-acid glycosphingolipids of chicken erythrocytes, 40 \( \mu \text{g} \); Lanes 2–9, glycosphingolipid fractions isolated from chicken erythrocytes, 0.5–2 \( \mu \text{g/lane} \).

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Compound A is easily identified from the NMR literature [17], mass spectrometry data, and the present COSY data, as globoside (GalNAc\(\beta\)3Gal\(\alpha\)4Gal\(\beta\)4Glc\(\beta\)1Cer). The terminal GalNAc\(\beta\)3 is thus clearly separated from internal ones by the H1/H2 connectivity seen at 4.509/3.75 ppm (see Table 1), as opposed to the corresponding values seen e.g. for the GalNAc\(\beta\)3 of the Forssman pentaglycosylceramide (4.511/4.03 ppm) [17]. Furthermore, the anomeric resonances of the Gal\(\alpha\)4 residues stemming from globoside and the Forssman pentaglycosylceramide are seen as expected at 4.796 ppm and 4.781 ppm, respectively, whereas the terminal GalNAc\(\alpha\)3 of the Forssman pentaglycosylceramide is readily found at 4.692 ppm, overlapping with another GalNAc\(\alpha\)3 anomeric resonance from the novel B compound. The H1/H2 connectivities arising from the GalB4Glc\(\beta\)1 segments of these two compounds are also clearly revealed as seen in the right panel of Fig. 4.

From the mass spectrometry data discussed above it was concluded that the four-sugar compound has the sequence HexNAc-HexNAc-Hex-Hex with both HexNAc residues being 3-linked. Inspection of Fig. 5B shows that the novel compound contains one \(\alpha\)- and one \(\beta\)-HexNAc at ppm values (4.699 ppm and 4.594 ppm, respectively) consistent with their identity being a terminal GalNAc\(\alpha\)3 and an internal GalNAc\(\beta\)3. The H1/H2 connectivity of the GalNAc\(\beta\)3 residue is only consistent with a penultimate position for this sugar residue (Table 1). Furthermore, a 4-linked GalNAc can be excluded since the preceding Gal\(\beta\)4 residue would in this case be expected to display an H1/H2 connectivity around 4.21/3.23 ppm as in e.g. gangliotetraosylceramide (Gal\(\beta\)3GalNAc\(\beta\)4GalB4Glc\(\beta\)1Cer) [18]. However, no such connectivity is observed in Fig. 4. The two remaining residues (Hex-Hex) are most likely represented by a GalB4Glc\(\beta\)1 segment.
where the anomeric resonances, however, are obscured by the corresponding resonances from globoside and the Forssman pentaglycosylceramide. The sequence of compound B is thus concluded to be GalNAc$_a$3GalNAc$_ß$3Gal$_a$4Glc$_ß$1Cer.

Further inspection of Fig. 5A reveals three minor additional anomeric resonances yet to be assigned to specific residues: the one to lowest field is an $α$-signal at 4.827 ppm revealing an H2 signal at 3.75 ppm thus indicating a Gal$_a$4 residue [19]; the second one is a $ß$-signal at 4.645 ppm having the H2 resonance at 3.40 ppm, which identifies this sugar as a GlcNAc$_ß$3 residue which probably stems from a small percentage of neolactohexaosylceramide (the closeby intensity seen at 4.66/3.37 ppm in Fig. 4 is an artefact due to the residual HDO resonance); the third one is also a $ß$-signal seen partially overlapping the GalNAc$_ß$3 resonances stemming from globoside and the Forssman pentaglycosylceramide on the low-field side. This resonance is more clearly seen in the COSY spectrum and the H1/H2 connectivity at 4.524/4.03 ppm identifies also this sugar as an internal GalNAc$_ß$3 residue. The observation that the Gal$_a$4 H1 resonance is shifted almost 0.3 ppm to lower field is suggestive of an extended galabiosylceramide based four-sugar structure. Galabiosylceramide has previously been identified in chicken erythrocytes [15] and characterized by NMR [20]. In such a case the H1/H2 connectivity of Gal$_ß$1 is expected around 4.08/4.29 ppm, which is exactly where a cross-peak of appropriate intensity is seen in Fig. 4. In the absence of a clearly distinguishable anomeric resonance that may conclusively identify the sugar in the fourth position, it may be inferred that a GalNAc$_a$3 residue whose resonances completely overlap those of the corresponding residue.
in the Forssman antigen is the only viable assumption, thus suggesting that the final structure should be GalNAcß3Gal-NAcß3Galß4Glcß1Cer (compound C). From a mass spectrometry point of view this section also explains why only one four-sugar compound could be detected.

NMR data for the C:hexa fraction was also obtained (not shown), but due to the low amount of material in this case, the spectral quality was rather poor. However, the signal to noise ratio was sufficient enough in order to clearly identify the Forssman pentaglycosylceramide, along with a second compound having three HexNAc residues whose identities are Galß4Glcß1, which leaves the third Hex that most likely can be assigned to a second Galß4 residue. Taken together these data allows the structure to be identified as GalNAcß3Galß4Glcß1Cer and GalNAcß3Galß4Glcß1Cer (compound E). This novel amide and GalNAc fraction C:hexa by the presence of the Forssman pentaglycosylceramide region (Fig. 6B and C, lanes 1 and 3, marked with ** in 6A). In addition, the F4ab fimbriae and the F4ab-fimbriated bacteria (Fig. 6, lanes 1, 2 and/or 5, marked with * in 6A). In contrast, a specific binding of the F4ab fimbriae and the F4ab-fimbriated bacteria to some fast-migrating glycosphingolipids in the acid fractions was observed (Fig. 6B and C, lanes 2 and 4, marked with ~ in 6A). The two residues closest to the ceramide are most likely Galß4Glcß1, which leaves the third Hex that most likely can be assigned to a second Galß4 residue. Taken together these data allows the structure to be identified as GalNAcß3Galß4Glcß1Cer and GalNAcß3Galß4Glcß1Cer (compound E). This novel amide and GalNAc fraction C:hexa by the presence of the Forssman pentaglycosylceramide region (Fig. 6B and C, lanes 1 and 3, marked with ** in 6A). In addition, the F4ab fimbriae and the F4ab-fimbriated bacteria to some fast-migrating glycosphingolipids in the acid fractions was observed (Fig. 6B and C, lanes 2 and 4, marked with ~ in 6A).

The Forssman pentaglycosylceramide was obtained (see below), GalNAcß3GalNAcß3Galß4Glcß1Cer and GalNAcß3GalNAcß3Galß4Glcß1Cer are the tetra- and hexaosylceramides recognized by the F4 fimbriae, and the GalNAcß3GalNAcß3Galß4Glcß1Cer are the tetra- and hexaosylceramides recognized by the F4 fimbriae. However, since the terminal trisaccharide of this tetraglycosylceramide is identical with the terminal trisaccharide of the non-binding Forssman pentaglycosylceramide, a binding of F4 fimbriae to GalNAcß3GalNAcß3Galß4Glcß1Cer is less likely.

### Binding of F4 fimbriae and F4-fimbriated E. coli to glycosphingolipids of pig intestinal epithelium

Thereafter, the binding of F4ab, F4ac and F4ad fimbriae and F4-fimbriated E. coli to mixtures of total acid and non-acid glycosphingolipids isolated from mucosal scrapings of newborn piglet and adult pig intestines was tested. No binding of the F4ad fimbriae or F4ad-expressing E. coli to the glycosphingolipids of pig intestinal epithelium occurred (not shown). In contrast, a specific binding of the F4ab fimbriae and the F4ab-fimbriated bacteria to some fast-migrating glycosphingolipids in the acid fractions was observed (Fig. 6B and C, lanes 2 and 4, marked with * in 6A). In the non-acid fractions, a compound migrating in the monoglycosylceramide region was recognized by both the F4ab and the F4ad-expressing E. coli and the bacterial cells (Fig. 6, lanes 1, 2 and/or 5, marked with ** in 6A). In addition, the F4ab fimbriae and the F4ab-expressing E. coli recognized a compound migrating in the triglycosylceramide region (Fig. 6B and C, lanes 1 and 3, marked with *** in 6A).

| Table 1. Summary of glycosphingolipid structures identified by NMR in the 4–6 sugar region of fractions C:tetra-I, C:tetra-II, and C:hexa in chicken erythrocytes (the identity of the anomeric and other ring proton resonances are given in the NMR spectra shown in Figs. 4 and 5). |
| --- |
| **Structure** | **Trivial name** | **VI** | **V** | **IV** | **II** | **I** |
| A | Globoside | GalNAcß3 | Galß4 | Gallß4 | Glcß1 | Cer |
| H1 | 4.509 | 4.796 | 3.244 | 4.165 |
| H2 | 3.75 | 3.76 | 3.43 | 3.03 |
| H3 | 3.48 |
| B | Forssman pentaglycosylceramide | GalNAcß3 | GalNAcß3 | Galß4 | Gallß4 | Glcß1 | Cer |
| H1 | 4.699 | 4.594 | — | — |
| H2 | 4.10 | 3.95 |
| H3 | 3.55 | 3.44 |
| C | GalNAcß3 | GalNAcß3 | Galß4 | Gallß4 | Glcß1 | Cer |
| H1 | ~4.70 | 4.524 | 4.827 | 4.077 |
| H2 | 4.03 | 3.75 | 3.29 |
| H3 | 3.48 |
| D | Extended x2 | GalNAcß3 | GalNAcß3 | Gallß4 | GlcNAcß3 | Galß4 | Glcß1 | Cer |
| H1 | 4.70 | 4.596 | ~4.26 | 4.65 | ~4.26 | — |

*Chemical shift values are in some cases not given due to low intensities and severe resonance overlap. doi:10.1371/journal.pone.0023309.t001
In order to characterize the F4ab-binding acid glycosphingolipids of newborn piglet intestinal epithelium, this fraction was analyzed by TLC-FAB-MS (Figure S3). Thereby, the more slow-migrating F4ab-binding glycosphingolipid was tentatively identified as sulfated dihexosylceramide, while the fast-migrating F4ab-binding glycosphingolipid was tentatively identified as sulfated monohexosylceramide.

To confirm the identity of the sulfated glycosphingolipids recognized by the F4ab fimbriae, the binding of these fimbriae to a panel of reference sulfated glycosphingolipids was next evaluated (Fig. 7, and summarized in Table 2). Here the F4ab fimbriae recognized sulfatide (SO3-3Galß1Cer) with variant ceramide composition (Fig. 7, lanes 1–3), along with binding to sulf-lactosylceramide (SO3-3Galß4Glcß1Cer; lane 4), while no binding to sulf-gangliotetraosylceramide (SO3-3Galß3GalNAcß4Galß4Glcß1Cer; lane 5) or cholesterol-sulfate (not shown) occurred.

Thus, the F4ab-binding acid glycosphingolipids of piglet small intestinal epithelium were identified as sulfatide and sulf-lactosylceramide. When using reference glycosphingolipids, the F4ab fimbriae bound to sulfatide with both sphingosine and phytosphingosine long-chain bases, and both hydroxy and non-hydroxy fatty acids, i.e. the ceramide composition did not influence the binding.

Isolation and characterization of the F4ab/F4ac fimbriae binding monoglycosylceramide from pig small intestinal mucosa

The binding-active monoglycosylceramide was isolated by HPLC of the total non-acid glycosphingolipid fraction from adult pig small intestinal mucosa, and the preparative procedure was monitored by binding of radiolabeled F4ab and F4ac fimbriae on thin-layer chromatograms. Pooling of the F4ab/F4ac binding monoglycosylceramide fractions yielded 5.2 mg (denoted fraction P:mono).

Characterization of fraction P:mono identified galactosylceramide (Galß1Cer) with sphingosine and hydroxy 24:0 fatty acid as the binding-active component. This conclusion is based on the following observations:

I) On thin-layer chromatograms the binding-active monoglycosylceramide migrated as a distinct band at the lower margin of the monoglycosylceramide region (Fig. 8B–D, lane 3).

II) The negative ion FAB mass spectrum of fraction P:mono (Fig. 8E) had a major molecular ion at m/z 826 identifying a monohexosylceramide with sphingosine and hydroxy 24:0 fatty acid. A major ceramide ion at m/z 664, obtained by elimination of the carbohydrate unit, was also present (not shown). Thus, the glycosphingolipid was identified as a monohexosylceramide with sphingosine and hydroxy 24:0 fatty acid.

IV) The proton NMR spectrum of fraction P:mono (data not shown) revealed two anomeric proton resonances: one belonging to Galß1Cer at 4.066 ppm (60%), a shift consistent with the presence of hydroxy fatty acid, and the other belonging to Glcß1Cer with non-hydroxy fatty acids at 4.113 ppm (40%) [22]. Both species were found to contain a sphingosine base in line with mass spectrometry data.

Using the same techniques the non-binding monoglycosylceramide fractions displayed in Fig. 8, lanes 2, 4 and 5 were characterized as glucosylceramide (Glcß1Cer) with mainly sphingosine and hydroxy 24:0 fatty acid, glucosylceramide with sphingosine and hydroxy 16:0 fatty acid and phytosphingosine with hydroxy 24:1 fatty acid together with galactosylceramide with phytosphingosine with hydroxy 16:0-24:0 fatty acids, respectively (data not shown).
Thus, of the monoglycosylceramides from pig intestine the F4ab- and F4ac-fimbriae bound to galactosylceramide with a distinct preference for the species with sphingosine and hydroxy 24:0 fatty acid.

Isolation and characterization of the F4ab fimbriae binding non-acid triglycosylceramide of pig small intestinal mucosa

The F4ab binding triglycosylceramide was isolated by chromatography on an Iatrobeads column, and the fractions obtained were tested for F4ab binding activity using 125I-labeled F4ab fimbriae. The fractions obtained were pooled into four fractions according to thin-layer chromatographic resolution and binding of F4ab fimbriae (Fig. 9A, lanes 2–5). The fraction designated fraction Ptri:1 (1.7 mg; lane 2) had no F4ab binding activity. Fractions Ptri:II (0.9 mg; lane 3) and Ptri:III (1.0 mg, lane 4) were recognized by the F4ab fimbriae, while fractions Ptri: IV (0.3 mg; lane 5) was non-binding.

Structural characterization of fraction Ptri:III demonstrated that the F4ab binding glycosphingolipid was globotriaosylceramide (Galα4Galβ4Glcβ1Cer) with phytosphingosine and hydroxy 24:0 fatty acid. This conclusion is based on the following observations:

I) The binding-active compound migrated in the triglycosylceramide region on thin-layer chromatograms (Fig. 6, lanes 1 and 3).

II) Fractions Ptri:I, Ptri: II and Ptri:III were analyzed by LC-ESI/MS using polyamine columns (to be published separately). Thereby, the non-binding fraction Ptri:I gave a series of [M-H]+ ions at m/z 1022, m/z 1078, m/z 1106, and m/z 1134, corresponding to a

Figure 7. Binding of F4ab fimbriae to reference glycosphingolipids. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of 125I-labeled F4ab fimbriae (B). The lanes were: Lane 1, sulfatide (SO3-3Galβ1Cer) with d18:1-24:1 ceramide, 4 μg; Lane 2, sulfatide with d18:1-h16:0 ceramide, 4 μg; Lane 3, sulfatide with t18:0-h24:0 ceramide, 4 μg; Lane 4, sulf-gangliotetraosylceramide (SO3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer), 4 μg. The glycosphingolipids visualized with anisaldehyde in (A) are marked with Roman numbers, and the corresponding glycosphingolipid structures are given below the chromatograms. doi:10.1371/journal.pone.0023309.g007

Table 2. Summary of glycosphingolipid binding specificities of F4ab, F4ac and F4ad fimbriae.

| No. | Trivial name                               | Structure                          | F4ab* | F4ac* | F4ad* |
|-----|-------------------------------------------|------------------------------------|-------|-------|-------|
| I.  | Chicken erythrocyte glycosphingolipids    |                                    |       |       |       |
| 1.  | Galactosylceramide                        | Galβ1Cer                           | +++   | +++   | –     |
| 2.  | GalNAcβ3GalNAcβ3Galβ4Glcβ1Cer             | ++                                 | +++   | +     | –     |
| 3.  | GalNAcβ3GalNAcβ3Galβ4Glcβ3Galβ4Glcβ1Cer  | ++                                 | +++   | +     | –     |
| II. | Porcine intestinal glycosphingolipids     |                                    |       |       |       |
| 1.  | Galactosylceramide                        | Galβ1Cer                           | +++   | +++   | –     |
| 2.  | Sulfatide                                 | SO3-3Galβ1Cer                      | +++   | +     | –     |
| 3.  | Sulf-lactosylceramide                     | SO3-3Galβ4Glcβ1Cer                 | +++   | –     | –     |
| 4.  | Globotriaosylceramide (t18:0-h24:0)       | Galβ4Glcβ1Cer                      | +++   | –     | –     |
| III. | Reference glycosphingolipids              |                                    |       |       |       |
| 1.  | Lactosylceramide (t18:0-h16:0-h24:0)      | Galβ4Glcβ1Cer                      | +++   | +++   | +     |
| 2.  | Galabiosylceramide                        | Galβ4Glcβ1Cer                      | +++   | –     | –     |
| 3.  | Isogalactosylceramide                     | Galβ3Galβ4Glcβ1Cer                 | –     | +     | +     |
| 4.  | Gangliotriaosylceramide                   | GalNAcβ4Galβ4Glcβ1Cer              | –     | –     | +     |
| 5.  | Gangliotetraosylceramide                  | Galβ3GalNAcβ4Galβ4Glcβ1Cer         | –     | –     | +     |
| 6.  | Neolactotetraosylceramide                 | Galβ4Glcβ3Galβ4Glcβ1Cer            | –     | –     | +     |

F4ab denotes bindings obtained with both F4ab fimbriae and F4ab-fimbriated E. coli, F4ac bindings obtained with both F4ac fimbriae and F4ac-fimbriated E. coli, and F4ad bindings obtained with both F4ad fimbriae and F4ad-fimbriated E. coli.

Binding is defined as follows: +++ denotes an intense and highly reproducible staining when 4 μg of the glycosphingolipid was applied on the thin-layer chromatogram, + denotes an occasional staining while – denotes no binding even at 4 μg.

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glyco-sphingolipid with three Hex and sphingosine with non-hydroxy 16:0, 20:0, 22:0, and 24:0 fatty acids, respectively (Fig. 9C). A \([\text{M-H}]^+\) ion at \(m/z\) 1022, indicating a trihexosylceramide with sphingosine with non-hydroxy 16:0 fatty acid, was also present in the mass spectra of fractions P:tri:II and P:tri:III (Fig. 9D and E). However, these F4ab binding fractions also had a major peak at \(m/z\) 1168 indicating a glycosphingolipid with three Hex and phytosphingosine and hydroxy 24:0 fatty acid. This was confirmed by the series of fragment ions at \(m/z\) 1006 (Y2; 1168-Hex), \(m/z\) 844 (Y1; 1168-Hex-Hex) and \(m/z\) 682 (Y0; 1168-Hex-Hex-Hex) obtained by MS2 of the ion at \(m/z\) 1168 (Fig. 9F).

The proton NMR spectrum of the F4ab-fimbriae binding fraction P:tri:III (not shown) revealed a single anomeric signal at 4.78 ppm (Gal\(\alpha\)4), several overlapping signals centered around 4.26 ppm (Gal\(\beta\)4) and three overlapping signals in the range 4.19–4.21 ppm (Glc\(\beta\)1), respectively, readily identifying this compound as globotriaosylceramide (Gal\(\alpha\)4Gal\(\beta\)4Glc\(\beta\)1Cer) through comparison with previously published spectra [23].

The binding of F4ab fimbriae to a panel of reference glycosphingolipids related to globotriaosylceramide was thereafter evaluated, in order to further investigate the requirements for the
globotriaosylceramide binding by these fimbriae (Fig. 10). Here, the F4ab fimbriae bound to globotriaosylceramide with phytosphingosine and hydroxy or non-hydroxy fatty acids (lanes 2–4), and to lactosylceramide with phytosphingosine and hydroxy fatty acids (lane 6). However, isoglobotriaosylceramide (lane 5) was not recognized although it had phytosphingosine and hydroxy fatty acids, and no binding to globotriaosylceramide or lactosylceramide with sphingosine and non-hydroxy fatty acids (lane 1 and lane 7) occurred.

### Binding of F4 fimbriae and F4-fimbriated E. coli to reference glycosphingolipids

To further investigate the structural requirements for F4 fimbriae glycosphingolipid recognition, the binding of F4-expressing E. coli, and F4 fimbriae, to a number of reference glycosphingolipids related to the binding-active compounds was next examined. The results are exemplified in Figs. 11 and 12, and summarized in Table S1. Here, lactosylceramide (Galβ4Glcβ1Cer; Fig. 11, lane 4, upper band, and Fig. 12, lane 3, upper band) with phytosphingosine and hydroxy fatty acid was recognized by all three subtypes of F4. However, for the remaining binding-active compounds, the recognition profiles differed between the F4 variants. Galactosylceramide (Galβ1Cer; Fig. 11, lane 3, upper band, and Fig. 12, lane 2, upper band) was the preferred ligand for F4ac fimbriae and F4ac-expressing bacteria, and binding to other glycosphingolipids, as e.g. sulfate (SO₃-Galβ1Cer; Fig. 11, lane 1, upper band), by the F4ac subtype occurred very occasionally. Galactosylceramide was also recognized by the F4ab fimbriae and F4ab-fimbriated bacteria. The F4ab subtype also bound to sulfate (Fig. 11, lane 1, upper band) and to globotriaosylceramide (Galβ2Galβ4Glcβ1Cer; Fig. 11, lane 6). In addition, galabiaosylceramide (Galβ2Galβ1Cer; Fig. 11, lane 5, upper band) was recognized by F4ab. Finally, the most divergent subtype was the F4ad, where both the fimbriae and the bacterial cells bound to gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer; Fig. 11, lane 7, and Fig. 12, lane 6) and gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer; Fig. 11, lane 3, lower band, and Fig. 12, lane 7), and occasionally to isoglobotriaosylceramide.

### Figure 10. Binding of F4ab fimbriae to non-acid reference glycosphingolipids

Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of [125I]-labeled F4ab fimbriae (B), F4ac fimbriae (C), and F4ad fimbriae (D). The lanes were: Lane 1, sulfatide (SO₃-Galβ1Cer) of human intestine with t18:0-h24:0 ceramide, 4 µg; Lane 2, glucosylceramide (Galβ4Glcβ1Cer) of bovine brain from Sigma-Aldrich with d18:1-16:0-24:0 ceramide, 4 µg; and gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0-24:0 ceramide, 4 µg; Lane 3, galabiaosylceramide (Galβ2Galβ1Cer) of bovine brain from Sigma-Aldrich with d18:1-h18:0-h24:0 ceramide, 4 µg, and gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) of mouse intestine with t18:0-h16:0 and h24:0 ceramide, 4 µg; Lane 4, lactosylceramide (Galβ4Glcβ1Cer) of dog intestine with t18:0-h16:0-h24:0 ceramide, 4 µg, and B5 pentaglycerosylceramide (Galβ3Galβ4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 µg; Lane 5, galactosylceramide (Galβ1Cer) of porcine kidney with d18:1-16:0-24:0 ceramide, 4 µg, and globotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) of human neutrophils with d18:1-16:0 and 24:0 ceramide, 4 µg; Lane 6, gangliotriaosylceramide (Galβ4Glcβ1Cer) (synthetic) with d18:1-16:0-18:0 ceramide, 4 µg, and P1 pentaglycerosylceramide (Galβ4Galβ4Glcβ1Cer) of guinea pig erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 µg. The glycosphingolipids visualized with anisaldehyde in (A) are marked with Roman numbers, and the corresponding glycosphingolipid structures are given below the chromatograms.

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### Figure 11. Binding of F4 fimbriae to reference glycosphingolipids

Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of [125I]-labeled F4ab fimbriae (B), F4ac fimbriae (C), and F4ad fimbriae (D). The lanes were: Lane 1, sulfatide (SO₃-Galβ1Cer) of human intestine with t18:0-h24:0 ceramide, 4 µg, and globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0-24:0 ceramide, 4 µg; Lane 2, glucosylceramide (Galβ4Glcβ1Cer) of bovine brain from Sigma-Aldrich with d18:1-16:0-24:0 ceramide, 4 µg, and globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0-24:0 ceramide, 4 µg; Lane 3, galabiaosylceramide (Galβ2Galβ1Cer) of bovine brain from Sigma-Aldrich with d18:1-h18:0-h24:0 ceramide, 4 µg, and gangliotetraosylceramide (Galβ3GalNAcβ4Galα4Glcβ1Cer) of mouse intestine with t18:0-h16:0 and h24:0 ceramide, 4 µg; Lane 4, lactosylceramide (Galβ4Glcβ1Cer) of dog intestine with t18:0-h16:0-h24:0 ceramide, 4 µg, and B5 pentaglycerosylceramide (Galβ3Galβ4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 µg; Lane 5, galactosylceramide (Galβ1Cer) of porcine kidney with d18:1-16:0-24:0 ceramide, 4 µg, and globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer) of human neutrophils with d18:1-16:0 and 24:0 ceramide, 4 µg; Lane 6, gangliotriaosylceramide (Galβ4Glcβ1Cer) (synthetic) with d18:1-16:0-18:0 ceramide, 4 µg, and P1 pentaglycerosylceramide (Galβ4Galβ4Glcβ1Cer) of guinea pig erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 µg. The glycosphingolipids visualized with anisaldehyde in (A) are marked with Roman numbers, and the corresponding glycosphingolipid structures are given below the chromatograms.

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Galectin 3 (Galβ4Glcβ1Cer; Fig. 12, lane 5) and neolactotetraosylceramide (Galβ4Glcβ4Glcβ1Cer) of human intestine with t18:0-h24:0 ceramide, 4 µg; Lane 7, gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) of rat intestine with t18:0-h22:0-h24:0 ceramide, 4 µg; Lane 7, gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) of guinea pig erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 µg. The glycosphingolipids visualized with anisaldehyde in (A) are marked with Roman numbers, and the corresponding glycosphingolipid structures are given below the chromatograms.

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### Glycosphingolipid recognition of F4ab deletion mutant fimbriae

To evaluate the relative roles of different F4ab subunits in the glycosphingolipid interaction, fimbriae were isolated from recombinant bacteria expressing F4ab fimbriae with deletions of FaeH, FaeE and FaeC. In glycosphingolipid binding assays, the binding patterns obtained with these deletion mutant fimbriae were identical to that of the native F4ab fimbriae (Figure S4). In
addition, bacteria expressing F4ab fimbriae with deletions of FaeH, FaeI and FaeJ bound in the same manner as the F4ab-positive wild type *E. coli* (Fig. S4). Thus, the minor F4ab subunits FaeH, FaeI and FaeJ are not involved in F4ab glycosphingolipid recognition.

**Discussion**

Microbes often utilize cell surface glycoconjugates for target cell recognition, and the binding of bacterial pathogens to host cell glycoconjugates can be mediated by both fimbrial and non-fimbrial adhesins. Here we have investigated the glycosphingolipid recognition of F4 fimbriae, a major virulence factor of enterotoxigenic *E. coli* causing neonatal and post-weaning diarrhea. The glycosphingolipid binding preferences of the three F4 variants are summarized in Table 2.

In the initial studies using a panel of erythrocyte glycosphingolipids from different species we found a distinct binding of all three F4 variants to three chicken erythrocyte non-acid glycosphingolipids, migrating as mono-, tetra- and hexa-glycosyceramides. Previous studies of chicken erythrocyte glycosphingolipids have demonstrated the presence of galactosylceramide, lactosylceramide and the Forssman pentaglycosylceramide in this source [15]. The binding of F4ab and F4ac to reference galactosylceramide, and galactosylceramide isolated from porcine intestine, suggested that the binding-active monoglycosylceramide from chicken erythrocytes was galactosylceramide. It should here be noted that no binding of the F4ad fimbriae to galactosylceramide occurred.

The F4ab/ac/ad-binding tetra- and hexa-glycosyceramides of chicken erythrocytes were characterized by mass spectrometry and proton NMR as GalNAcβ3Galα3Gal4GlcBl1Cer and GalNAcβ3Galα3Gal4GlcNacβ3Galβ4GlcBl1Cer, both of which are novel glycosphingolipid structures. GalNAcβ3Galα3Galβ4GlcBl1Cer has previously only been characterized in the Forssman (GalNAcβ3Galα3Gal4GlcBl1Cer) and the iso-Forssman (GalNAcβ3Galα3Gal4GlcBl1Cer) pentaglycosylceramides [24,25]. No binding of the three F4 variants to the Forssman pentaglycosylceramide was obtained, suggesting that the binding epitope of the chicken tetra- and hexa-glycosyceramides was the GalNAcβ3Galα3Galβ4GlcBl1Cer moiety.

When using the erythrocyte panel a binding of F4ad fimbriae and F4ad-fimbriated bacteria, but not the F4ab or F4ac variants, to gangliotriaosylceramide (Galβ3Galα3Gal4GlcBl1Cer), the major non-acid glycosphingolipid of guinea pig erythrocytes was also obtained.

However, there was no correlation between the hemagglutination patterns of the F4 variants and the glycosphingolipid bindings obtained, suggesting that the glycosphingolipids are not involved in the hemagglutination process.

Next, the binding of the F4 variants to glycosphingolipids from the small intestinal epithelium of neonatal and adult pigs was evaluated. Here, no binding of the F4ad fimbriae or F4ad-fimbriated *E. coli* to the acid or non-acid glycosphingolipids of pig intestine was obtained. However, for F4ab and F4ac two distinct binding patterns were observed. The F4ac fimbriae and the F4ac-expressing *E. coli* selectively bound to galactosylceramide with sphingosine and hydroxy 24:0 fatty acid. This compound was also recognized by the F4ad fimbriae and the F4ad-fimbriated bacteria.

Binding of F4ab fimbriae to galactosylceramide in piglet ileal mucus has been described previously [26]. In addition, we found that the F4ab fimbriae and F4ad-fimbriated bacteria also bound to sulfatide, sulf-lactosylceramide and globotriaosylceramide with phytosphingosine and hydroxy or non-hydroxy fatty acids.

Finally, when the binding to reference glycosphingolipids from other sources was tested all three serotypes bound to lactosylceramide (Galβ4GlcBl1Cer) with hydroxy ceramide. F4ab also recognized galabioosylceramide (Galβ4GallBl1Cer), in line with the binding to porcine intestinal globotriaosylceramide (Galβ4GallBl1Cer). In addition, a selective binding of the F4ad fimbriae and the F4ad-fimbriated *E. coli*, but not the F4ab or F4ac subtypes, to reference gangliotriaosylceramide (Galα3Galβ4GlcBl1Cer) and gangliotetraosylceramide (Galβ3Galα3Gal4GlcBl1Cer) was observed,
and occasionally F4ad also bound to isoglobotriaosylceramide (Galβ3Galβ4Glcα1Cer) and neolactotetraosylceramide (Galβ4Glcα1
Neuα3Galβ4Glcβ1Cer).

The major subunit FaeG is considered to be the adhesive subunit, since sequence comparison of the genes encoding the minor and major fimbrial subunits of the F4ab and F4ac variants showed that the differences between these variants are confined to the faeG gene [9]. This was supported by the fact that removal of all minor fimbrial subunits by treatment of the F4 fimbriae with 2 M urea at 55°C did not affect the adhesive properties of the fimbriae [7]. In general, the F4ab and F4ac variants showed more similarities in their glycosphingolipid recognition patterns compared to the F4ad variant (Table S1 and Table 2). Comparative analysis of the FaeG sequences from various F4ab, F4ac and F4ad antigenic variant strains revealed that the amino acid sequences of the F4ab and F4ac variants had a higher degree of homology (92%), compared to the homology between the F4ad and F4ac (88%) [27]. Within each F4 serotype, 96–100% homology in the FaeG amino acid sequence was observed. Interestingly, an unusually large carbohydrate binding site, consisting of an enlarged Ig-domain with insertion of two α-helices and two β-strands, is found in the crystal structure of the F4ad variant of FaeG [28].

The repertoire of glycosphingolipids recognized by the F4ab fimbriae is the most diverse, since this fimbrial subtype binds to sulfatide, sulf-lactosylceramide, galactosylceramide, lactosylceramide, galabiaosylceramide, globotriaosylceramide and the two galactosylceramide and globotriaosylceramide with hydroxy ceramides, but do not bind either galactosylceramide or sulfatide. That the Galα4Gal motif is indeed recognized by the F4ab fimbriae is shown by the parallel binding of this fimbrial subtype to galabiaosylceramide. However, for the F4ab fimbriae a substitution of the terminal Gal with a βGalNAc in 3-position is not tolerated since globoside is non-binding.

Binding of uraerophasic F1c-fimbriated E. coli to galactosylceramide and globotriaosylceramide with hydroxy ceramides, but not to sulfatide, has also been reported [36].

Sulfatide recognition is also a common theme in microbial adherence, and binding to sulfatide has been reported for e.g. colonization factor antigen CS6 from human ETEC, Mycoplasma pneumoniae, Bordetella pertussis, 987P-fimbriated E. coli, and Helicobacter pylori [37–42]. Interestingly, the subtype of the heat-stable enterotoxin of E. coli which is primarily associated with diarrhea in piglets (the STb subtype) also binds to sulfatide [43].

Finally, the binding pattern of F4ad, with recognition of lactosylceramide with hydroxy ceramide, isoglobotriaosylceramide, neolactotetraosylceramide, gangliotetraosylceramide and gangliotriaosylceramide, has previously been reported for several bacteria, both pathogens and members of the indigenous flora [44]. It should, however, be noted that no binding of the F4ad fimbriae and the F4ad-fimbriated E. coli to the porcine intestinal glycosphingolipids was obtained, suggesting that the glycosphingolipid binding of F4ad has no relevance for bacterial attachment to the porcine small intestinal epithelium.

The objective of present study was to characterize the erythrocyte glycosphingolipids, and the glycosphingolipids of porcine small intestinal epithelium, recognized by the F4ab, F4ac and F4ad fimbriae, with the ultimate goal to create a platform for synthesis of anti-adhesive substances. Candidate receptors for adherence to the epithelial cells of porcine intestine were identified as galactosylceramide, sulfatide, sulf-lactosylceramide and globotriaosylceramide for F4ab, galactosylceramide only for F4ac, while no binding of F4ad to the porcine intestinal glycosphingolipids samples occurred. Still, F4ad binding neolactotetraosylceramide has previously been identified in porcine intestinal epithelial cells [13]. Whether the presence of the glycosphingolipids recognized by the F4 variants is related to the differences in the adhesiveness of F4 subtypes to pig intestinal brush border membranes is currently investigated.

Furthermore, two candidate porcine intestinal glycoprotein receptors for the F4 fimbriae have previously been identified, i.e. a pair of mucin-type dialglycoproteins (210 kDa or 240 kDa) recognized by F4ab and F4ac, and a 74-kDa transferrin glycoprotein recognized by F4ab (reviewed in [2,45]). Characterization of the glycans of these F4 binding glycoproteins is an important next step.

**Materials and Methods**

**Bacterial strains, culture conditions and labeling**

The wild type F4ab-positive E. coli strains C585-80 (serotype O8: K87; H19:F4ab, LT+), the wild type F4ac-positive E. coli strain IMM01 (serotype O149:K91:F4ac, LT+, STb+) [46], and the wild type F4ad-positive E. coli C1360-79 (serotype O8:H10:F4ad) [15] were cultured on BHI agar plates (Oxoid, Basingstoke, Hampshire, England) at 37°C for 18 h [7]. E. coli K12 PLoS ONE | www.plosone.org 12 September 2011 | Volume 6 | Issue 9 | e23309
containing the F4ab-encoding plasmid pDB88-8, and the mutant derivatives of pDB88-8, were grown on BHI agar plates, supplemented with ampicillin (100 µg/ml) at 37°C over night. The FaeH- mutant strain contains a stop codon in faeH, whereas the FaeI mutant strain contains a deletion in faeI, and the FaeJ mutant strain contains a frameshift deletion in faeJ. The resulting plasmids were named pDB88-141 (FaeH- mutant), pDB88-85 (FaeI- mutant) and pDB88-84 (FaeJ- mutant) [7]. After growing, the bacteria were harvested by centrifugation and suspended in PBS (phosphate-buffered saline, pH 7.3). The concentration of bacteria in the suspensions was determined by measuring the optical density at 606 nm (A606). An optical density of 1 equals 10⁶ bacteria per milliliter, as determined by counting colony forming units.

For metabolic labeling, the culture plates were supplemented with 10 µl ³⁵S-methionine (400 µCi; Amersham Pharmacia Biotech). Bacteria were harvested, washed three times in PBS, and resuspended in PBS containing 2% (w/v) bovine serum albumin (MP Biomedicals, LLC, Illkirch, France) 0.1% (w/v) NaN₃ and 0.1% (w/v) Tween 20 (BSA/PBS/TWEEN) to a bacterial density of 1×10⁶ colony forming units/ml. The specific activity of bacterial suspensions was approximately 1 cpm per 100 bacteria.

**Fimbrial preparations**

The F4ab-, F4ac- and F4ad wild type fimbriae and F4ab mutant fimbriae were purified as described by Van den Broeck et al. [48]. In short, the wild type F4 positive E. coli were grown in tryptone soy broth (DIFCO Laboratories, Biotrading, Bierbeck, Belgium) at 37°C for 18 h while shaking at 85 rpm. The FaeH-, FaeI- and FaeJ-mutant strains were grown in tryptone soy broth, supplemented with ampicillin (100 µg/ml) [7]. Bacteria were harvested by centrifugation (3500 rpm, 30 min, 4°C), washed and suspended in PBS. Subsequently, F4 fimbriae were isolated by homogenizing the bacterial suspension using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany) at 24,000 rpm for 15 min keeping the suspension on ice. Next, the bacteria were pelleted by centrifugation for 20 min at 10,000×g at 4°C, and the supernatant was further purified by centrifugation for 40 min at 20,000×g at 4°C. The solubilized fimbriae were subsequently precipitated with 40% ammonium sulfate. After centrifugation, the pellet was dissolved and dialyzed overnight against PBS. The protein concentration of the isolated fimbriae was determined using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Bornem, Belgium).

**¹²⁵I-labeling**

Aliquots of 100 µg of protein were labeled with ¹²⁵I, using Na ¹²⁵I (100 mCi/ml; Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the IODO-GEN protocol of the manufacturer (Pierce, Rockford, IL), giving approximately 2×10⁶ cpm/µg protein.

**Reference glycosphingolipids**

Total acid and non-acid glycosphingolipid fractions were isolated as described [49]. Individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns and by HPLC, and identified by mass spectrometry [50] and 1H-NMR spectroscopy [18]. Galactosylceramide (Galβ1Cer) of bovine brain with d18:1-h13:0-h24:0 ceramide was purchased from Sigma-Aldrich, St. Louis, MO. Synthetic galabiosylceramide (Galβ1-Galβ1Cer) was a kind gift from late Dr. Göran Magnusson, Land University, Sweden.

**Thin-layer chromatography**

Thin-layer chromatography was done on aluminum- or glass-backed silica gel 60 high performance thin-layer chromatography plates (Merck, Darmstadt, Germany). Glycosphingolipid mixtures (10–80 µg) or pure glycosphingolipids (0.5–4 µg) were applied to the plates, and if not otherwise stated, eluted with chloroform/methanol/water (60:35:8, by volume). Chemical detection was done with anisaldehyde [51].

**Chromatogram binding assay**

Binding of radiolabeled fimbriae and bacteria to glycosphingolipids on thin-layer chromatograms was done as described previously [37]. Dried chromatograms were dipped in diethylether/n-hexane (1:5 v/v) containing 0.5% (w/v) polyisobutylmethacrylate for 1 min. To diminish background binding the chromatograms were blocked with BSA/PBS/TWEEN for 2 h at room temperature. Then the plates were incubated with ¹²⁵I-labeled fimbriae (1–5×10⁶ cpm/ml) or ³⁵S-labeled bacteria (1–5×10⁶ cpm/ml) diluted in BSA/PBS/TWEEN for another 2 h at room temperature. After washing six times with PBS, and drying, the thin-layer plates were autoradiographed for 12 h using XAR-5 x-ray films (Eastman Kodak, Rochester, NY).

**Isolation of the F4 fimbriae binding slow-migrating non-acid glycosphingolipids from chicken erythrocytes**

Acid and non-acid glycosphingolipids were isolated from chicken erythrocytes by standard methods [49]. Briefly, the erythrocytes were lyophilized and then extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and 1:9, by volume, respectively). The material obtained was subjected to mild alkaline hydrolysis and dialysis, followed by separation on a silicic acid column. Acid and non-acid glycosphingolipid fractions were obtained by chromatography on a DEAE-cellulose column. In order to separate the non-acid glycolipids from alkali-stable phospholipids, this fraction was acetylated and separated on a second silicic acid column, followed by deacetylation and dialysis. Final purifications were done by chromatographies on DEAE-cellulose and silicic acid columns.

Part of the non-acid glycosphingolipids (8.4 mg) were separated on a 10 g Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) column, eluted with chloroform/methanol/water 65:23:4 by volume), 32×0.5 ml, followed by chloroform/methanol/water 60:35:8 by volume), 66×0.5 ml, and finally chloroform/methanol/water 40:40:12 by volume), 15 ml. The fractions obtained were tested for binding of F4ab and F4ad fimbriae using the chromatogram binding assay. The F4-binding glycosphingolipid migrating in the tetraglycosylceramide region was eluted in fractions 65–71. Pooling of these fractions yielded 0.9 mg (designated fraction C:tetra-I). The binding-active compound migrating in the hexaglycosylceramid region eluted in fractions 91–97, and after pooling of these fractions less than 0.1 mg was obtained (designated fraction C:hexa-I). Proton NMR revealed that fraction C:tetra-I was a mixture of four glycosphingolipids. This fraction was therefore further separated on a 1 g Iatrobeads column, eluted with chloroform/methanol/water 60:33:8 by volume), 10×0.5 ml. Pooling of the F4-binding fractions gave 0.2 mg (designated fraction C:tetra-II).

**Isolation of the F4ab/F4ac fimbriae binding non-acid monoglycosyleramide from pig small intestinal mucosa**

Acid and non-acid glycosphingolipids were isolated from mucosal scrapings from the small intestine of an adult pig as described [49].
Part of the non-acid glycosphingolipids (150 mg) was thereafter separated by HPLC on a 2.1×25 cm Kromasil 5 Silica column (particle size 5 μm; Phenomenex, Torrence, CA), eluted with a linear gradient of chloroform/methanol/water 90:10:1 to 60:35:8 (by volume) during 180 min with a flow rate of 2 ml/min. Aliquots of each 2 ml fraction were analyzed by thin-layer chromatography, and the fractions positive for anisaldehyde staining were further tested for binding of F4ab fimbriae, using the chromatogram binding assay. Glycosphingolipids migrating as monoglycosylceramides were collected in tubes 41–72, and the F4ab fimbriae binding compound was collected in tubes 62–65. Pooling of tubes 62–65 yielded 5.2 mg, and this fraction (designated fraction P:mono) was used for structural characterization.

Isolation of the F4ab fimbriae binding non-acid triglycosylceramide from pig small intestinal mucosa

The subfractions containing glycosphingolipids migrating as diglycosylceramides and below, from the separation described above, were pooled giving 26.4 mg. This material was separated on an Iatrobeads (Iatrobeads 0RS-0060; Iatron Laboratories, Tokyo) column (10 g), eluted with chloroform/methanol/water 65:23:4 (by volume), 4×5 ml, followed by 36×1 ml, and finally chloroform/methanol/water 60:35:5 (by volume), 1×10 ml. Compounds migrating in the triglycosylceramide region were collected in fractions 14–29. The fractions were pooled into four fractions according to thin-layer chromatographic resolution and binding of F4ab fimbriae. The fraction designated fraction P:tri:III (1.7 mg) had no F4ab binding activity. Fractions P:tri:II (0.9 mg) and P:tri:III (1.0 mg) were recognized by the F4ab fimbriae, while fractions P:tri:IV (0.3 mg) was non-binding.

Thin-layer chromatography - negative ion FAB mass spectrometry

The acid glycosphingolipid fraction from newborn piglet small intestinal mucosa (50 μg) was separated on aluminium-backed silica gel 60 HPTLC plates using chloroform/methanol/water 60:35:8 (by volume) as solvent system. Several thin-layer chromatograms were developed in parallel for bacterial binding, chemical staining with anisaldehyde and thin-layer chromatography - negative ion FAB mass spectrometry (TLC-FAB-MS), respectively [52]. Mass spectrometry was performed with a ZAB-2F/TF mass spectrometer (VG Analytical, Manchester, UK). For TLC-FAB-MS, a movable FAB probe (VG Analytical) was used. The thin-layer plates with separated glycosphingolipids were cut into 6 mm wide strips, and after mounting on the probe, a layer of triethanolamine (Fluka, Buchs, Switzerland) was applied using a soft roller. Negative ion FAB mass spectra were produced by Xe atoms, 8 kV. Four scans per mm of the thin-layer plate were recorded.

Negative ion FAB mass spectrometry

Negative ion FAB mass spectra of purified glycosphingolipids were recorded on a JEOL SX-102A mass spectrometer (JEOL, Tokyo, Japan). The ions were produced by 6 keV xenon atom bombardment, using triethanolamine (Fluka, Buchs, Switzerland) as matrix, and an accelerating voltage of −10 kV.

Endoglycoceramidase digestion and LC/MS

Endoglycoceramidase II from Rhodococcus spp. [53] (Takara Bio Europe S.A., Gennevilliers, France) was used for hydrolysis of glycosphingolipids. Brieﬂy, 50 μg of the F4ab fimbriae binding non-acid glycosphingolipid fractions from chicken erythrocytes (fractions Ccteta and Cchexa) were resuspended in 100 μl 0.05 M sodium acetate buffer, pH 5.0, containing 120 μg sodium cholate, and sonicated briefly. Thereafter, 1 μl of endoglycoceramidase II was added and the mixture was incubated at 37 °C for 48 h. The reaction was stopped by addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters, Milford, MA). The eluant containing the oligosaccharides was dried under nitrogen and under vacuum.

The glycosphingolipid-derived oligosaccharides were analyzed by capillary-LC/MS and MS/MS as described [16]. In brief, the oligosaccharides were separated on a column (200×0.180 mm) packed in-house with 5 μm porous graphite particles (Hypercarb, Thermo Scientific), and eluted with an acetonitrile gradient (A: 8 mM ammonium bicarbonate; B: 100% acetonitrile). The saccharides were analyzed in the negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA).

ESI/MS and ESI/MS/MS of native glycosphingolipids

The glycosphingolipids (dissolved in methanol) were analyzed on an LTQ linear quadrupole ion trap mass spectrometer by static nano-ESI/MS at −1.7 kV, using type F gold-coated needles (Micromass/Waters, Milford, MA). Full-scan (m/z 380–2000, 2 microscans, maximum 100 ms, target value of 30 000) was performed, followed by data dependent MS2 scans (2 microscans, maximum 100 ms, target value of 100 000) with normalized collision energy of 30%, an isolation window of 3 μ, an activation q = 0.25, and an activation time of 30 ms.

Proton NMR spectroscopy

1H NMR spectra were acquired on a Varian 600 MHz spectrometer at 30°C. Samples were dissolved in dimethyl sulfoxide/D2O (98:2, by volume) after deuterium exchange. Two-dimensional double quantum-filtered correlated spectroscopy (DQF-COSY) spectra were recorded by the standard pulse sequence [54].

Supporting Information

Figure S1 Purified wild type and deletion mutant F4 fimbriae. The protein preparations were separated by SDS-PAGE (12%), and stained by Coomassie Brilliant Blue R-250. The lanes were; Lane 1, F4ab fimbriae with deletion of FaeH, 5 μg; Lane 2, F4ab fimbriae with deletion of FaeI, 5 μg; Lane 3, F4ab fimbriae with deletion of FaeJ, 5 μg; Lane 4, wild type F4ab fimbriae, 5 μg; Lane 5, wild type F4ac fimbriae, 5 μg; Lane 6, wild type F4ad fimbriae, 5 μg; Lane 7, molecular weight marker (kDa). (TIFF)

Figure S2 ESI/MS/MS of the native fraction C:Tetra-II from chicken erythrocytes. Above the spectrum is an interpretation formula representing the molecular species with t18:0-h16:0 ceramide. (TIFF)

Figure S3 TLC-FAB-MS of the acid glycosphingolipids from newborn piglet small intestinal mucosa. (A) Thin-layer chromatogram stained with anisaldehyde. Acid glycosphingolipids (50 μg) of the epithelial cells of neonatal piglet small intestine were separated on aluminum-backed HPTLC plates using chloroform/methanol/water 60:35:8 (by volume) as solvent system. (B) Autoradiogram obtained by binding of 125I-labeled F4ab fimbriae to the acid glycosphingolipids of newborn piglet small intestine. (C–E) Reconstructed curves of selected ions of NeuGe-GM3 (C), sulfated dihexosylceramide (D) and sulfated glycosphingolipid Recognition by F4 Fimbriae 1

Supporting Information

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Supporting Information

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Supporting Information
monohexosylceramide (E), representing the successive detection of different ceramide species. TLC-FAB-MS was performed as described [52], using a ZAB-2F/1F mass spectrometer (VG Analytical, Manchester, UK). Four scans per mm of the thin-layer plate were recorded. The scanning of the thin-layer plate started at the bottom (to the left in the figure) and was run approximately 60 mm upwards, giving in total about 250 scans. After scanning approximately 40 mm of the thin-layer chromatogram, i.e. at the level of the more slow-migrating F4ab-binding glycosphingolipid, peaks appeared that corresponded to molecular ions of sulfated dihexosylceramide. In scans 157–164, the peak at m/z 956, corresponding to the species with d18:1-h16:0, was dominating, and in scan 165 the species with t18:0-16:0 (m/z 958) dominated. The following scans had peaks corresponding to d18:1-h16:0 (m/z 940), d18:1-h22:0 (m/z 1040), and d18:1-h24:0 or t18:0-24:1 (m/z 1068) ceramides. At the level of the more fast-migrating F4ab-binding compound (scans 168–211) molecular weight ions of sulfated monohexosylceramide were obtained. Here, ions corresponding to sulfated monohexosylceramide with d18:1-h16:0 (m/z 794), d18:1-h16:0 (m/z 778), d18:1-h22:0 (m/z 878), d18:1-h24:0 or t18:0-24:1 (m/z 906), and d18:1-h24:0 (m/z 890) were found. Thus, the more slow-migrating F4ab-binding glycosphingolipid was tentatively identified as sulfated dihexosylceramide, while the fast-migrating F4ab-binding binding glycosphingolipid was tentatively identified as sulfated monohexosylceramide. (TIF)

Figure S4  Binding of deletion mutant F4ab fimbriae to glycosphingolipids. Thin-layer chromatograms after chemical detection by anisaldehyde (A and J), and autoradiograms obtained corresponding to sulfated monohexosylceramide with d18:1-h16:0 and d18:1-24:0 ceramide, 4 Galß1Cer) with d18:1-h18:0-h24:0 ceramide, 4 Galß3Gal and anomeric configurations. Biochemistry 22: 2676–2687.

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Author Contributions
Conceived and designed the experiments: ST AC EC JA Ā. Performed the experiments: AC EV JB JĀ ST. Contributed reagents/materials/analysis tools: MEB EC ST. Wrote the paper: ST AC JĀ.
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