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Escherichia coli K88 Interaction with IgA Oligosaccharides

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ABSTRACT

Diarrhea from enterotoxigenic *Escherichia coli* (*E. coli*) expressing the K88 fimbrial adhesin causes high morbidity and mortality among newborn and weaned piglets. K88 fimbrial adhesins are surface filaments with lectin activity that recognize specific glycoconjugates (glycoproteins or glycolipids) on the surface of intestinal cells. Carbohydrates that compete for adhesion attachment could serve as an alternative for disease prevention. In this study, IgA, IgG and IgM oligosaccharides were tested to inhibit the adhesion of *E. coli* K88 to piglet mucins. Immunoglobulins were isolated from porcine serum by hydrophobic interaction chromatography (HIC) and purified by affinity chromatography. *In vitro* K88 adhesin interacts specifically with IgA oligosaccharides, but not with carbohydrates of IgG or IgM. Also IgA oligosaccharides partially inhibit the adherence of K88 strain to porcine intestinal mucins.

Keywords: Affinity purification, IgA oligosaccharides, *E. coli* K88 adhesin, piglet mucins, inhibition of adhesion.

INTRODUCTION

Protein-carbohydrate interactions appear to play a critical role in the adherence of pathogens to eukaryotic cells (Karlsson, 2001). Many microbial pathogens including those responsible for enteric infections, exploit oligosaccharides that are displayed on the surface of host cells as receptor for toxin and adhesin (Paton et al., 2006). *E. coli* expressing the K88 adhesin on their surface is a common cause of diarrhea in newborn and weaned piglets (Wilson and Francis, 1986). K88 fimbrial adhesins are filamentous surface appendages with lectin activity that allows *E. coli* to attach to specific glycoconjugates (receptors) on porcine intestinal mucins and epithelial cells (Grange et al, 1998). Adhesion appears to prevent bacterium removal by intestinal peristalsis, facilitating colonization of the small intestine. K88 fimbriae can specifically

adhere to sialoglycoproteins, intestinal mucin-type glycoproteins, and neutral glycosphingolipids via α . and β-linked galactose structures (Grange et al, 1999; Grange et al., 1998; Blomberg et al., 1993; Payne et al., 1993; Neeser et al., 1986; Gibbons et al., 1975). Thus, oligosaccharides that compete for microorganism-adhesins could provide an alternative for disease prevention.

The importance of immunoglobulins (Igs) in protecting new borne against diarrhea reflects not only antigen-antibody interactions but also possible interactions with the complex carbohydrates associated to immunoglobulin heavy chains (Owusu et al, 2002; Araujo et al, 2000; Gomez et al, 1998; Cravioto et al, 1991; Wold et al., 1990; Cruz et al, 1988; Rutter et al., 1976). In fact, the carbohydrate portion of human IgA binds to pathogens to prevent their adhesion to intestinal cells (Cravioto et al, 1991) and suggests that carbohydrates of IgA could serve as a possible target for prevention of infection (Rajan et al, 1999). Carbohydrates that specifically interfere with the initial microorganism interaction could hinder the disease process. This concept is supported by studies in which sugars or other synthetic receptor analogs that block the binding of microorganisms to cells and prevent microbial invasion (Karlsson et al., 2001; Rajan et al, 1999; Rosenstein, 1994; Karlsson et al, 1992; Schaeffer et al, 1984). The objective of the present study was to determine whether K88 adhesin could interact with porcine serum IgA oligosaccharides and compete for mucin sites.

MATERIALS AND METHODS
Materials
 Sepharose CL 6B was purchased from GE Healthcare (Uppsala, Sweden). Radial immunodiffusion VET-RID kits, goat anti-pig for IgA, IgG, and IgM were from Bethyl Labs (Montgomery, TX. USA). Divinylsulfone (DVS) activated agarose was from Kem-En-Tek, (Copenhagen, Denmark). BSA-Galα(1-3)Gal was from Glycorex, (Sweden). All other reagents were analytical grade from Sigma Aldrich (St. Louis, Mo, USA).

Sample preparation
Porcine blood was collected in sterile containers at the bleeding line of a certified slaughterhouse located in Hermosillo, México. Blood was allowed to coagulate at 25ºC and serum separated by decanting, followed by centrifugation at 24 000 g for 15 min at 4ºC. The fat layer was removed at this time and aliquots of serum stored at -40 ºC until use.

IgA, IgM and IgG purification
Porcine immunoglobulins were isolated in a single step by hydrophobic interaction chromatography (HIC) using a highly acetylated Sepharose (HA-Sepharose) synthesized according to Vázquez-Moreno et al. (1992). HA-Sepharose was packed at 1.5-mL/min flow rate, to obtain a 2 mL bed volume (10 x 0.5 cm) column. HA-Sepharose was equilibrated with five bed volumes of 0.5 M Na_2SO_4, 10 mM MOPS, pH 7.6 (Buffer A) and samples of 5 mL of porcine serum (adjusted to 0.5 M Na_2SO_4, 10 mM MOPS, pH 7.6) loaded onto column. The gel was washed with Buffer A to remove unadsorbed proteins that correspond to albumin fraction. Igs were eluted with 10 mM MOPS Buffer, pH 7.6 (Buffer B). Following elution, HA-Sepharose was cleaned with two bed volumes of 4 M guanidine hydrochloride (guanidine-HCl), pH 7.6; washed with five bed volumes of distilled water and re-equilibrated with Buffer A (Ramos-Clamont et al., 2006). All chromatographic procedures were monitored

by absorbance at 280 nm (Spectronic 21 spectrophotometer, Milton Roy, USA) and analyzed for protein content by Bradford (1976) using bovine serum albumin (BSA) as a standard.

Each type of immunoglobulin (IgA, IgM or IgG) was purified by affinity chromatography using an anti-IgA, anti-IgM or anti-IgG agarose matrix according to Vázquez-Moreno et al. (1993). Forty mg of anti-pig for IgA, IgM, or IgG coupled individually to 2 g of DVS activated agarose (Kem-En-Tek, Copenhagen, Denmark). Gels were packed in columns (4 x 1 cm), equilibrated with PBS, pH 7.2 and loaded with 10 mg of isolated Igs fraction (in PBS, pH 7.2) from hydrophobic interaction chromatography. Unbound proteins were washed with PBS and the specific immunoglobulin eluted from the column with 0.02 M glycine HCl, pH 3.6. Elution fractions were immediately neutralized with 0.1 M Tris buffer, pH 8.0.

Quantitative radial immunodiffusion

The relative amount of IgA, IgM or IgG in unabsorbed and adsorbed fractions, as well as in serum samples were determined by radial immunodiffusion (RID) according to Fahey and McKelvey (1965). Quantitative RID kits containing goat anti-pig for IgA, IgM or IgG were used according to vendor (Bethyl Labs, Montgomery TX. USA). Plate wells were loaded with reference standards of Igs or with 160 μg of protein from each chromatographic fraction. Immunoglobulin concentration of unknown samples was determined by locating their precipitation diameter on a semi log plot. Different concentrations of pig Igs were used as standard. Precipitation diameters were measured with a VET-RID reader and an ocular device Finescale (Horscale, Labconco, USA). Duplicate samples were analyzed for immunoglobulin content at three independent times.

Polyacrylamide gel electrophoresis

Protein fractions were analyzed by electrophoresis using 8 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) according to Laemmli (1970). Gels were stained with 1 % Coomasie blue in 30% methanol and 10% acetic acid.

Immunoblotting assay

Assays were performed as described by Towbin et al. (1979). Unadsorbed and elution fractions (25 μg) were separated on 8 % SDS-PAGE gels, under reducing conditions (Laemmli, 1970) and transferred to nitrocellulose membranes at 2.5 mA/cm^2 for 40 min (Semi-dry blotter, Bluchler, Labconco, USA). IgA was detected with anti-pig IgA (α chain specific, 1:1000 dilution) raised in goat, followed by incubation with anti-goat IgG peroxidase (as secondary antibody, 1:3000 dilution). Procedures for detection of IgG and IgM were similar but using the corresponding antibody. Color reactions were developed by H_2O_2 addition and 3,3′diaminobenzidine.

Piglet mucin isolation

Intestinal sections (duodenum, jejunum and ileum) were obtained from 9 healthy newborn piglets (17 days-old) according to the method of Miller and Hoskins (1981). Briefly, small intestines were taken immediately after slaughter, flushed with phosphate buffered saline A (PBS-A; 10 mM phosphate, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/mL NaN_3, 10 mM EDTA and 2 mM iodoacetamide), intestinal sections were opened longitudinally and cut into 20 cm segments. Mucus was gently removed with a glass slide and suspended in four volumes of ice-cold PBS-A. The suspension was shaken for 1 h at 4°C and centrifuged for 30 min at 15,000 g to remove particulate matter. Mucus

was isolated from the extracts by dual precipitation with ice-cold ethanol, freeze-dried and suspended in 10 mM HEPES-Hanks buffer at a concentration of 0.5 mg/ml. Mucin content was determined by the alcian blue method of Hall et al., (1980). Bovine submaxilary mucins were used as standard. All assays were done in duplicate.

Adhesion assays

The *E. coli* K88 strain, kindly donated by Dr. Carlos Eslava from Universidad Nacional Autonoma de Mexico, was maintained as frozen stocks. Overnight cultures were grown in nutritive broth on a shaker at 37°C. After harvesting, bacteria cells were washed twice in Hanks balanced salt solution (HBSS, Sigma, St. Louis, MO. USA) and suspensions adjusted to $\approx 2x10^9$ CFU ml^{-1} by optical density equal to 1.0 at 600 nm (Jin et al., 2000).

E. coli K88 was biotin labeled by mixing 0.9 mL of culture (2×10^9 CFU ml^{-1}) with 0.1 ml of dimethyl sulfoxide containing 0.2 mg of NHS-LC-Biotin (Pierce, Rockford, Ill. USA). Labeling of bacteria was performed in the presence of 100 mM lactose to protect lectin-binding sites (Ruhl et al., 1996). After 3 h of incubation, at room temperature with occasional mixing, labeled bacteria were washed 4 times in HBSS to remove free NHS-LC-Biotin.

For blotting assays, serial two fold dilutions containing from 1 μg/μL to 1 ng/μL of protein were prepared for IgA, IgM, IgG, piglet mucins and control glycoproteins. Aliquots of 1 μL were applied to nitrocellulose (Ruhl et al., 1996). Glycoproteins used as positive controls included fetuin (F), asialofetuin (AF), ovalbumin (OV), porcine gastric mucin (PGM), asialo porcine gastric mucin (APGM), bovine submaxilary mucin (BSM)

and a neoglycoprotein BSA-Gal α(1-3)Gal prepared by conjugation of Galα(1-3)Gal to BSA (Glycorex, Sweden). Untreated BSA was used as negative control.

Membranes spotted with glycoproteins were blocked overnight in TTBS (0.15 M NaCl, 20 mM Tris HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% sodium azide and 0.05 % Tween 20, pH 7.8) containing 3% defatted milk and 1 μg/mL of avidin (Wood and Warnke, 1981). After washing with TTBS, membranes were overlaid with biotinylated bacteria to a final concentration of approximately 5×10^8 CFU per ml in the same buffer. Following overnight incubation at 4°C, membranes were washed to remove unbound bacteria and developed with avidin-alkaline phosphatase to detect bacterial adhesion. Similarly, bacterial adhesion was also tested using denatured and dissociated IgA, IgM or IgG. Igs samples (25 μg) were reduced, separated by SDS-PAGE and transferred to nitrocellulose (Ruhl et al., 1996).

To test the inhibition of lectin-mediated adhesion of *E. coli* K88 *to* piglet mucin by IgA, samples containing 2 μg of duodenum jejunum and ileum, mucins, were spotted on nitrocellulose and incubated with $\approx 5 \times 10^8$ CFU/ml of labeled *E. coli* K88 (positive control). For inhibition studies, bacteria was mixed with different concentrations IgA (0.3-5 μg/ml) and pre-incubated for 45 min before overlaid to nitrocellulose membranes as described before.

Hemagglutination activity and carbohydrate inhibition were performed by the serial double-dilution method using piglet erythrocytes (Jaffe, 1980). Bacteria cells were washed and suspended in PBS pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Suspensions were adjusted to 10^9 CFU/mL. Piglet erythrocytes were suspended to 2% in

a PBS solution, pH 7.2. For agglutination activity, 25 µL of bacterial suspension were deposited on an agglutination plate, serially diluted and followed by the addition of 25 µL of erythrocytes. HT was defined as the inverse maximal dilution showing visible agglutination.

Inhibition assays were done by mixing 25 µL of appropriately diluted glycoprotein solutions with 25 µL of bacterial suspension. After 40 min incubation, 25 µL of 2 % erythrocyte suspension were added. Inhibition was determined as the concentration of glycoproteins that prevent 50% of the HT (Jaffe, 1980). Glycoproteins included F, AF, OV, PGM, APGM, BSM, porcine Igs (IgA, IgG and IgM), and piglet duodenum mucin. The initial stock concentration of glycoproteins was 1 mg/mL.

RESULTS

IgA purification

The immunoglobulin fraction (containing IgA, IgM, and IgG) was isolated from porcine serum by HIC as described by Ramos-Clamont et al., (2006), and IgA was purified from this fraction by affinity chromatography (Fig. 1). Fractions containing 10 mg of immunoglobulins were applied to anti-IgA column; unadsorbed proteins were washed with PBS pH 7.6 (Fig. 1, fraction A), while those interacting with the matrix were eluted with 0.02 M Glycine-HCl, pH 3.5 (Fig. 1 fraction B).

The IgA content in serum and chromatographic fractions was estimated by radial immunodiffusion (Table 1). Serum contained 2.2 ± 0.1 mg/mL of IgA; also 2.6 ±

0.1 and 20.2 ± 0.1 mg/ml of IgM and IgG respectively (data not shown). IgA remained in the adsorbed HIC fraction. IgA (2.2 ± 0.1 mg/mL) was purified in the adsorbed fraction of anti-IgA agarose. As expected the IgA content corresponded to 9-10% of total immunoglobulin fraction (Butler and Brown, 1996).
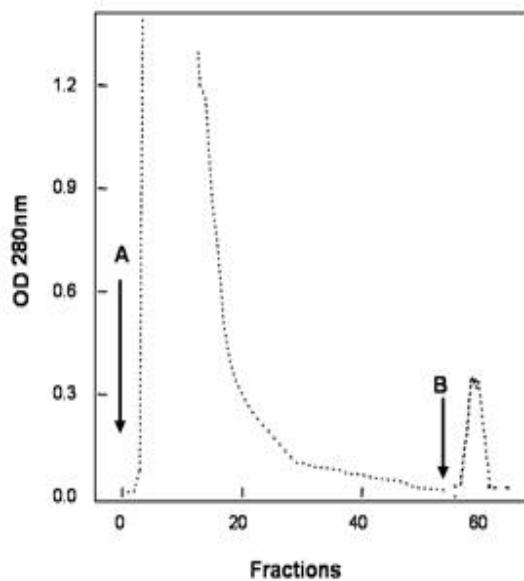
Figure 1: Elution of IgA purified from porcine immunoglobulins. Column containing immobilized anti-IgA agarose was equilibrated with PBS, pH 7.6 and porcine immunoglobulins applied. Unbound proteins remained in the flow through (A). Adsorbed proteins eluted (B) with Glycine-HCl, pH 3.5; flow rate 0.5 ml/min; fractions, 3.0 ml/tube.

Table 1. Immunoglobulin A content in chromatography fractions

	HIC			*Affinity Chromatography*	
	Total Protein (mg/mL)	IgA (mg/mL)		Total Protein (mg/mL)	IgA (mg/mL)
Serum	40.0 ± 0.3	2.2 ± 0.1[a]	Ig fraction	10 ± 0.1	2.7 ± 0.1
Unadsorbed (albumin fraction)	32.4 ± 0.2	ND	Unadsorbed (IgM, IgA, IgG)	9 ± 0.2	1.7 ± 0.2
Adsorbed (Ig fraction)	7.6 ± 0.3	2.2 ± 0.1	Adsorbed (IgA)	1 ± 0.1	1 ± 0.1

[a] Concentrations were estimated by RID according to Fahey and McKelvey (1965). Average value and standard deviation of six determinations
Ig: Immunoglobulin
ND: Not Detected

Adhesion assays

Serial two dilution samples containing purified IgA, IgM and IgG, piglet mucins and glycoproteins were immobilized on nitrocellulose and overlaid with biotin-labeled *E. coli* K88 for adhesion assays. K88 fimbrial adhesin bind to IgA at concentrations from 1 µg to 0.25 µg (Fig. 2A). In contrast, no bacterial adhesion was observed when IgG and IgM were spotted (data not shown). Bacterial adhesion was greater to duodenum mucins than to jejune or ileum mucins (Fig. 2B). Also bovine submaxilary mucin and asialofetuin were recognized (Fig 2C). No adhesion was observed with fetuin, porcine gastric mucin or ovalbumin (data not shown). In contrast adhesion of E. *coli* K88 was observed to as little as 1.9 ng of BSA-Gal α(1-3)Gal neoglycan. These results suggest that the galactosyl residue containing glycoproteins may serve as receptors for the lectin-like components of *E. coli* K88.

Bacterial adhesion to denatured IgA was also examined. IgA was reduced separated by SDS-PAGE (Fig. 3A) and transferred to nitrocellulose membranes (Fig. 3B). Membranes were incubated with *E. coli* K88 labeled with biotin. *E. coli* interacted with both BSA-Gal(α 1-3)Gal neoglycan and with the heavy chain of porcine IgA (Fig. 3B). Immunoglobulin heavy chain was confirmed with anti-pig IgA antiserum (Bethyl Labs, Montgomery, TX) as shown in figure 3C.
Mucins were spotted in nitrocellulose and overlaid with *E. coli* K88 bacteria that was pretreated with purified IgA (0.3 -5 µg/ml).
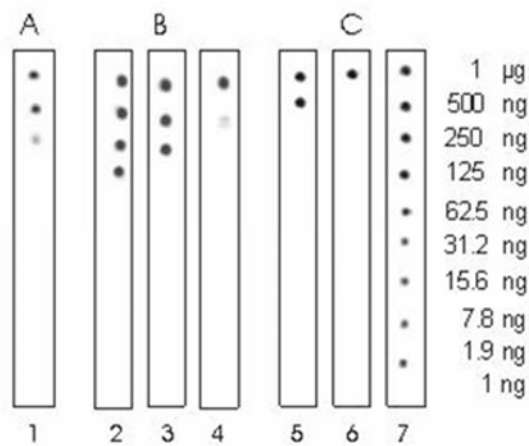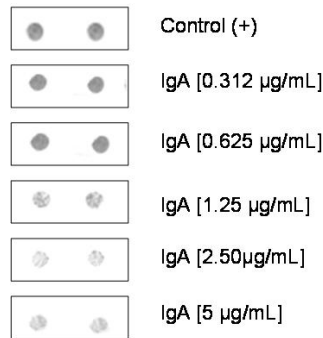
Figure 2: Lectin-mediated adhesion of *E. coli* K88 *to* immobilized glycoproteins. Proteins were applied in serial dilution (1 µg to 1 ng) on nitrocellulose. Membranes were then overlaid with biotinylated *E. coli* K88 incubated for 24 h and washed. Interactions were detected using avidin-alkaline phosphatase. A). Purified porcine IgA from serum (1); B). Piglet intestinal mucins from duodenum (2), jejunum (3), and ileum (4); C). Glycoproteins: asialofetuin (6), bovine submaxilary mucin (7) and BSA-Gal α(1-3)Gal neoglycan (8).
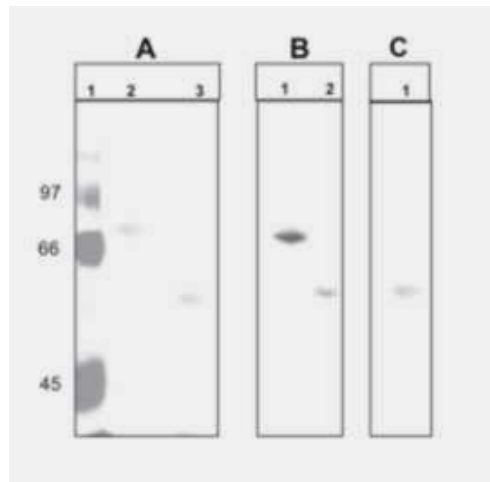
Figure 3: Detection of IgA heavy chain by bacterial overlay and immunoblotting. (A) SDS-PAGE of porcine serum IgA. Lane 1. Molecular weight standards; lane 2. BSA-Gal (α 1-3) Gal; lane 3. purified IgA. (B). Western blot of porcine IgA. Lane 1; BSA-Galα(1-3) Gal and lane 2 reduced and denatured IgA. Samples containing 25 µg of purified IgA were reduced, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were overlaid with biotinylated *E. coli K88* and the interaction developed with avidin-alkaline phosphatase to detect adherent bacteria. (C) Western blot of the IgA heavy chain (α). IgAα was detected using antiserum raised against anti-pig α
chain.

IgA was able to reduce bacterial adhesion to mucins in a dose-dependent manner (Figure 4).

Control (+)

IgA [0.312 µg/mL]

IgA [0.625 µg/mL]

IgA [1.25 µg/mL]

IgA [2.50 µg/mL]

IgA [5 µg/mL]

Figure 4. Partial inhibition of lectin-mediated adhesion of *E. coli* K88 *to* piglet mucin by porcine serum IgA. Samples containing 2 µg of piglet mucin were spotted on nitrocellulose and incubated with ≈ 5 x 10 ^{8} CFU/ml of biotin labeled *E. coli* K88 (Positive control). For inhibition studies, bacteria was mixed with the indicated concentrations of porcine serum IgA and incubated for 45 min before overlaying the nitrocellulose membranes. Interactions were revealed with avidin-alkaline phosphatase to detect adherent bacteria.

The bacterial adherence to the intestinal mucosa is a prerequisite in colonization, and therefore, considerable attention has been given to the factors related to adherence. The adhesive ability of an enteropathogen is usually assessed by determining the hemagglutinating ability, because the erythrocyte membrane is believed to possess similar receptors as those of the mucosal involved in bacterial adherence (Anantha et al., 2004; Nagayama et al., 1994, Cravioto et al., 1982). In addition, high correlation has been observed between agglutination of erythrocytes from different species caused by pili and production of enterotoxin by porcine, bovine, and human strains of *E. coli* (Meng et al., 1998; Evans et al., 1979, 1977; Jones and

Rutter, 1974, 1972). Thus piglet erythrocytes were used to test of hemagglutination caused by *E. coli* K88 (Table 2). Fimbrial bacterial agglutination was inhibited by piglet duodenum mucin (0.001 mg/mL) and porcine purified IgA (0.008 mg/mL), while Immunoglobulin fraction required 0.010 mg/mL). This strongly suggests that inhibition is due to IgA present in the total immunoglobulin fraction. Bovine submaxilary mucin was as effective as Igs. On the contrary, asialofetuin was required in greater concentration (8x) to inhibit agglutination.

Table 2. Inhibition of hemagglutination by IgA containing fractions

Inhibition of Hemagglutination	mg/mL[a]
Study fractions	
Piglet duodenum mucin	0.001
Porcine IgA	0.008
Porcine Igs (IgA,IgG,IgM)	0.010
Commercial glycoproteins	
Bovine submaxilary mucin	0.008
Asialofetuin	0.063
Porcine gastric mucin	N.I
Bovine serum albumin	N.I
Ovalbumin	N.I

(n = 9)
[a] Minimum concentration (mg/mL) required to inhibit a hemmaglutination titer of 8
N.I. No inhibition

DISCUSSION

Pathogen Gram-negative bacteria adhesion is mediated mainly by adhesins. Bacterial adhesins bind to host cell complex carbohydrates located at the mucosal epithelial cells leading to an infection (Sharon and Ofek, 2001). K88 fimbriae can specifically adhere to gut receptors via α and

ß-linked galactose structures (Grange et al., 1998; Blomberg et al., 1993; Payne et al., 1993; Neeser et al., 1986; Gibbons et al., 1975).

Among the strategies included to prevent diarrhea in animals is the use of probiotic bacteria or specific monoclonal antibodies directed against adhesins. The mechanisms, by which probiotics exert beneficial health effects, are not well understood, but appear to involve immune modulation of the host, production of antimicrobial compounds and competitive exclusion of probiotics against pathogens (Kirjavainen et al., 1998). Recent studies with *Lactobacillus gasseri* K7 found that this probiotic was effective in reducing *E. coli* adhesion to Caco-2 enterocytes, however was not able to do so in *ex vivo* conditions tested for pig jejunal tissue (Bogovic et al., 2006). Alternatively, products from fermented food, such as soybean Tempe have high inhibitory activity of K88 hemagglutination as a result of its ability to aggregate the ETEC K88 cells (Kiers et al., 2002). This inhibition of aggregation was also observed by Van der Broeck et al. (2000) with anti-K88 specific monoclonal antibodies.

In this study, we used non-specific IgA for the inhibition of *E. coli* K88 agglutination. Grange and Mouricout (1996) and Grange et al. (2002) reported that K88 adhesin binds to porcine serum transferrin (pST) which contains a biantennary complex N-glycan with the structures Gal β(1-4)GlcNAc β (1-2)Man-- linked to Asn-497 (Sharma et al., 1994). This glycan moiety is also found in human IgA (Mattu et al., 1998). Furthermore, we show that the oligosaccharide moiety of purified porcine serum IgA was recognized by *E. coli* K88 as well as the epitope Gal α(1-3)Gal attached to BSA. *E. coli* K88

interaction was specific to IgA oligosaccharides, since no bacterial attachment was observed to the major Igs (IgG or IgM) that are also glycosylated with complex carbohydrates (Butler and Brown, 1994).

Bacteria can carry different adhesins with distinct specificities giving cells flexibility to attach to different receptors (Karlsson, 2001; Guinee and Jensen, 1979). The three serologically distinguishable variants of K88 (K88ab, K88ac, and K88ad) present lectins with different but related carbohydrate specificity, as reflected in their hemagglutination patterns. K88ab and K88ac agglutinate porcine erythrocytes (Bijlsma and Frik, 1987) while K88ad did not (Guinee et al., 1979; Orskov, 1964).

Further studies are required to define the specificities of adhesins from pathogens in order to propose treatments with well-defined glycoproteins that possess structurally known glycans.

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