Screening of extracts from natural feed ingredients for their ability to reduce enterotoxigenic *Escherichia coli* (ETEC) K88 adhesion to porcine intestinal epithelial cell-line IPEC-J2

G. González-Ortiz a,*, R.G. Hermes a, R. Jiménez-Díaz b, J.F. Pérez a, S.M. Martín-Ortúe a

a Servei de Nutrició i Benestar Animal (SNiBA), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain
b Departamento de Biotecnología de Alimentos, Instituto de la Grasa (Consejo Superior de Investigaciones Científicas), Avda. Padre García Tejero, 4, Apto. 1078, 41012 Sevilla, Spain

**ARTICLE INFO**

Article history:
Received 3 June 2013
Received in revised form 24 July 2013
Accepted 25 July 2013

Keywords:
IPEC-J2 cell-line, Enterotoxigenic E. coli K88, Fimbriae, Natural feed ingredients, Anti-adhesive therapies, Colibacillosis

**ABSTRACT**

Enterotoxigenic *Escherichia coli* (ETEC) K88 is the most prevalent enteropathogen in weaned piglets, with the ability to express fimbria F4 and specifically attach to intestinal receptors in the young piglet. The prevention of ETEC K88 adhesion to the epithelium by interfering in this fimbria-receptor recognition provides an alternative approach to prevent the initial stage of disease. The aim of this study is to screen, among different feed ingredients (FI), their ability to reduce ETEC K88 attachment to the porcine intestinal epithelial cell-line (IPEC-J2). The selected FI consisted of products of a vegetable or dairy origin, and microbial by-products, which could be suitable to be included in piglet’s diet. Incubation of a mixture of each FI extract with the bacteria on IPEC-J2 monolayer was allowed. After washing with PBS to remove the non-adhered bacteria, the culture medium was added to grow the adhered bacteria and, simultaneously, to keep the cells alive. Then, the bacterial growth was monitored in a spectrophotometer reader for 12 h. Casein glycomacropeptide (CGMP), locust bean (LB), exopolysaccharide (EPS) and wheat bran (WB) reduced the number of attached ETEC K88 to IPEC-J2, but no anti-adhesive effect was found for soybean hulls, sugar-beet pulp, locust gum, fructooligosaccharides, inulin, mushroom, mannanoigosaccharides or the fermented product from *Aspergillus oryzae*. The linear analysis of dose responses demonstrated lineal activity (P < 0.0001) for CGMP, LB, EPS and WB. These in vitro results suggest CGMP, LB, EPS and WB as good candidates to be included in piglet’s diet with supported functional activity against colibacillosis.

**1. Introduction**

The ability of pathogens to survive and initiate infection depends on their ability to adhere to host cell-tissues. Adherence involves the interaction of complementary molecules on the surface of bacteria and the surface of the host epithelium. The overall specificity of a bacterium for a particular host is contingent on the presence of definitive oligosaccharide receptors (Mazariiego-Espinosa et al., 2010).

Enterotoxigenic *Escherichia coli* is the most problematic bacteria in the piglet’s weaning period, leading to large economic losses. Besides its ability to produce enterotoxin (Zhang et al., 2007), this bacterium also expresses F4 fimbrial adhesins (K88), providing a highly specific means for ETEC attachment to receptors located in the brush border of the epithelial mucosa (Jin and Zhao, 2000).
Therefore, anti-adhesive strategies have emerged by adhesin analogs, competitively blocking pathogen adherence to receptors (Lane et al., 2010), or by receptor analogs, which resemble host oligosaccharide receptors, thus interrupting the adherence process (Shao-Sweeney and Hutkins, 2009). Naturally occurring carbohydrates exhibited fimbriae-blocking activity in a variety of assays (Roberts et al., 2013; Wang et al., 2010) and represent a viable option for anti-adhesive therapies (Sharon, 2006). However, the complex microbial ecosystem of the digestive tract makes it difficult to show the likely positive effects given by a single feed ingredient (FI) in the diet. The use of in vitro assays using cell cultures may enhance the knowledge between bacteria-to-cell interactions (Brosnahan and Brown, 2012) and, in this particular case, the specific anti-adhesive role of the candidate products.

The aim of this study is to screen, among different FI, their ability to reduce ETEC K88 attachment to the intestinal porcine epithelial cell-line (IPEC-J2).

2. Materials and methods

2.1. Cell-culture growth

The IPEC-J2 cell-line (epithelial cells derived from cells isolated from the jejunum of a neonatal pig and maintained as a continuous culture) was kindly donated by Dr. Antony Bilkslager, from the College of Veterinary Medicine (North Carolina State University). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (GIBCO®, Ref. no.: 31313-028, Life Technologies; Spain), supplemented with insulin, transferrin, selenium and ethanolamine added as ITS solution (GIBCO®, Ref. no.: 41400-045, Life Technologies; Spain) and maintained in an atmosphere of 5% CO₂ at 37 °C until confluence. Cells were used between passages 85 and 91 and were routinely tested to be free of mycoplasma contamination.

The IPEC-J2 cells were cultured in 75 cm² flasks and 2 x 10⁵ cells/well were seeded into 96-well flat-bottom plates (Nunclon Delta Surface, Ref. no.: 167008, Thermo Scientific; Denmark) in 200 μl of culture media. Cells were allowed to adhere for 24 h in an atmosphere of 5% CO₂ at 37 °C. When confluence was confirmed after 24 h, cells were washed once with PBS and then 200 μl of CO₂-independent medium (GIBCO®, Ref. no.: 18045-054, Life Technologies; Spain) were added. Plates were kept in a heater at 37 °C without providing CO₂ for 24 h, until they were ready for the in vitro test.

2.2. Escherichia coli strains

Two different E. coli strains were used in this experiment. The first one was an ETEC K88 strain isolated from a colibacillosis outbreak in Spain (Blanco et al., 1997), a serotype (O149;K91:H10 [K-88/1L-1STB] that was kindly provided by the E. coli Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The other one was a non-fimbriated (NF) E. coli strain (F4-, F6-, F18-, L1-), ST1-, ST2+ Stx2e-) isolated from the feces of a post-weaning piglet and kindly provided by the Department of Animal Health and Anatomy of the Universitat Autònoma de Barcelona. ETEC K88 was cultured in unshaken Luria broth at 37 °C with the aim to maximize fimbriae expression (Snellings et al., 1997), while the NF E. coli was cultured in shaking media. Bacteria were serially cultured every 48 h, at least three times.

Bacterial cells of a 15 ml overnight culture (1700 × g, 5 min) were collected by centrifugation, the supernatants were removed, and PBS buffer was added to the cell pellet to achieve an optical density at 650 nm (OD₆₅₀nm) of 1 (approximately log 9 – 8.5 CFU/ml) and serially diluted to reach log 7–6.5 CFU/ml.

2.3. Feed ingredients extraction

The FI used are shown in Table 1, including eight ingredients of vegetal origin, one dairy protein and three microbial by-products. All ingredients were prepared as previously described (Becker and Galleti, 2008). Coarse ingredients, like wheat bran (WB), soybean hulls (SO), sugar-beet pulp (SBP) and mushrooms (MSH) were finely ground in an analytical grinder. All products were suspended in PBS to a solid-to-liquid ratio of 1:100 (w/v). Subsequently, the suspensions were sonicated three times for 30 s each and then centrifuged (460 × g, 5 min). The supernatants were filter-sterilized (0.2 μm filters, Ref. no. 28145-477, VWR; Spain) and stored at −20 °C until used in the in vitro test.

2.4. In vitro test with IPEC-J2

The in vitro test was performed by adapting the methodology described by Hermes et al. (2011). As a first step, IPEC-J2 cells were subjected to a 24 h adaptation period in 96-well flat-bottom plates with a CO₂-independent medium. Separately, a mixture (1:1) of each FI extract with each E. coli strain suspension was gently mixed in a 1.5 ml eppendorf, and 200 μl were immediately transferred to each well. The mixtures and cells were incubated for 30 min at 37 °C, allowing non-blocked bacteria to attach to cells. Wells were gently washed once with sterile PBS by hand-pipetting so as not to disturb the IPEC-J2 monolayer, in order to remove the non-adhered bacteria. Finally, 200 μl of CO₂-independent medium was added to allow for the growth of the adhered bacteria and to keep the cells alive. Bacterial growth at 37 °C was monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation; Sunnyvale, California, USA) by measuring the OD₆₅₀ nm at intervals of 10 min for 12 h. The FI screening was performed in two independent assays, in triplicate. Feed ingredients that showed the highest specific anti-adhesive properties (CGMP, LB and EPS), and also WB by our previous background, were subsequently submitted to a dose–response experiment in which doses of 1%, 0.1% and 0.01% were evaluated. Again, two independent assays were performed in triplicate.

2.5. Bacterial CFU counts and t₉₀ = 0.05 correlations

The perceptibility ranges of ETEC K88 and the NF E. coli were determined as correlations between OD₆₅₀ nm values and colony counts on Luria agar. For that, both bacterial
strains were serially diluted in Luria broth medium and the CFU/ml was determined by plate counting. The growth characteristics were determined at 37 °C for 12 h every 10 min by measuring the OD_{650 nm} in the microplate reader. The bacterial growth times, t_{OD=0.05} (h), were analyzed following the same procedure explained above. For ETEC K88, the fitted equation was \( y = -1.682x + 13.916 \) (R^2 = 0.989), and for the NF E. coli, the equation obtained was \( y = -1.0841x + 9.364 \) (R^2 = 0.975), where “y” corresponded to the t_{OD=0.05} and “x” to the log of CFU per well.

2.6. Statistical analyses

All statistical analyses were performed using SAS 9.2 (SAS Inc.; Cary, NC, USA). OD_{650 nm} data were processed by non-linear regression analysis using the non-linear P-NLIN procedure (Gauss-Newton method) following the equations described by Becker and Galleti (2008). From the time at which the bacterial growth reached an OD_{650 nm} of 0.05 (t_{OD=0.05} h), the log CFU was calculated for each FI using the previously described linear models. Values are presented as means ± SD. Significant differences among F1 were determined by one-way analysis of variance (ANOVA). The linear trends were used to compare the dose–response effect. Differences between means were tested by the Tukey–Kramer adjustment for multiple comparisons.

3. Results and discussion

The intestinal porcine epithelial cell-line (IPEC-J2) was isolated from a neonatal suckling piglet (Berschneider, 1989). It conserves the epithelial nature and can serve as a convenient in vitro model system for studying porcine-specific pathogenesis (Schierack et al., 2006).

The screening among a variety of FI revealed differences regarding their ability to specifically reduce ETEC K88 adhesion to IPEC-J2 (Fig. 1). Three particular ingredients, LB, CGMP and EPS, showed a clear ability to reduce the number of ETEC K88 adhered to the cells, being more significant in CGMP (P = 0.01) and EPS (P = 0.0004). However, this significant reduction was not observed with the NF E. coli, suggesting that their chemical composition is able to disrupt the fimbrria F4 and receptor recognition of ETEC K88 (Koh et al., 2008). On the other hand, none of the other FI extracts at 1% were clearly able to significantly reduce ETEC K88 attachment. Nonetheless, interestingly inulin reduced (P = 0.03) the NF E. coli adhesion to IPEC-J2, as it did similarly on natural porcine mucus (unpublished data). Gibson et al. (2005) briefly reviewed the anti-adhesive properties of this type of oligosaccharides against bacterial pathogens; because of that, it was included in the present study. No reduction in ETEC K88 attachment to the IPEC-J2 cell-line was found, but it is difficult to explain the persistent effect against the NF E. coli. It may be hypothesized that interference with other adhesins different from fimbrria F4 that are only present in the NF E. coli can take part in such response (Fig. 2).

Regarding the products of vegetable origin, LB reduced ETEC K88 adhesion, as compared to PBS (P = 0.02). The LB used in this study was obtained by mixing locust pods and seeds from the Ceratonia siliqua tree, which has been described to contain a mixture of galactomannans, non-starch polysaccharides and phenolic compounds (Papagiannopoulos et al., 2004). Whereas galactomannans have
demonstrated the ability to reduce ETEC K88 adhesion to IPEC-J2 (Badia et al., 2012), other authors have reported that phenolic compounds play a role in disrupting the bacterial ligand-epithelial receptor (Liu et al., 2008). Nevertheless, the heterogeneous composition of this product makes it difficult to elucidate the specific molecular basis of this interaction.

The dairy compound CGMP also promoted a significant reduction on ETEC K88 adhesion to IPEC-J2 \( (P = 0.01), \) which agrees with previous in vitro and in vivo studies (Hermes et al., 2011, 2012). In contrast to the results with NF E. coli, this specific recognition of CGMP to ETEC K88 was also seen later by using chronoamperometric measurements (Espinoza-Castañeda et al., 2013). CGMP contains three glycosilation sites with a heterogenous array of glycans, based on a core of Galβ(1 → 3)GalNAc- and NeuAc(2 → 5)Gal-, which could act as a potential receptor analog (Rhoades et al., 2005; Grange et al., 2002).

The EPS used in this work came from the natural fermentation process of green olive brines in which Lactobacillus pentosus and yeasts play a relevant role (Garrido-Fernández et al., 1997). Results showed that EPS was the most efficient at reducing adhesion of the ETEC K88 strain to the IPEC-J2 cell-line among all FI regarding PBS \( (P = 0.0004). \) In this sense, similar interferences of EPS in the E. coli adhesion to Caco-2 (Alp et al., 2010) and
porcine erythrocytes (Wang et al., 2010) have been described. However, previous investigations by our group conversely detected that EPS does not reduce the attachment of ETEC K88 at the porcine mucosal intestine (unpublished data). The recruitment of intestinal pathogens by the mucosal layer is a mechanical way to push out undesirable bacteria, avoiding receptor attachment (Bergstrom et al., 2010). Therefore, it seems that EPS may possess a dual function versatility depending on the in vitro test environment exposed.

The dose–response assay was performed with those feed ingredients (LB, CGMP and EPS) that showed clear anti-adhesive properties. We also included WB in the dose–response assay because our previous results showed that WB might reduce the ETEC K88 attachment to the mucosa of the ileum (Molist et al., 2009) and to IPEC-J2 (Hermes et al., 2011). WB is the outermost covering of wheat grain, which is rich in carbohydrates (40% of non-starch polysaccharides, 34% of starch) and protein (12%) (Palmarola-Adrados et al., 2005). All of these ingredients reduced linearly ($P < 0.0001$) ETEC K88 adhesion to IPEC-J2, including the WB. About the vegetable products, a concentration of 0.1% of WB and LB did not have any anti-adhesive ability as did 1% ($P > 0.05$). When all FL at 1% were compared in the screening assay, WB resulted in a poorer anti-adhesive capacity. However, the dose–response assay re-confirmed the previous results obtained by our research group. CGMP, as well as EPS, were efficient at 0.1%, but the activity was lost when tested at 0.01%. The dose–response assay allowed for checking the minimum FL concentration able to reduce in vitro bacterial adhesion although it is difficult to extrapolate these values under in vivo conditions and to recommend a final dietary dose.

In this study, the soluble extracts of CGMP and EPS resulted in the best feed ingredients to reduce ETEC K88 attachment to IPEC-J2, but the vegetable extracts from LB and WB were also able to prevent bacterial adhesion. Their promising results make them good candidates to be included in the piglet’s diet with supported functional activity against colibacillosis.

Conflict of interest

There are no conflicts of interest statements between the authors.

Acknowledgments

This work was supported by a public research project of the Spanish Ministry of Education and Science (project AGL 2009-0732). We thank Dr. Calvo of the UAB for culturing the strains. The authors also wish to acknowledge the companies that kindly provided the feed ingredients used: Armegol Hermanos S.A., Polygal S.A., Arla Foods and Molimen, S.L. The authors also wish to acknowledge the members of the Servei de Cultius Cel-lulars of the UAB, for continuous cell maintenance and the technical support (Dr. Paqui García, Fran Cortés and Silvia Rayo).

References


