Bromelain protects piglets from diarrhoea caused by oral challenge with K88 positive enterotoxigenic *Escherichia coli*

D S Chandler, T L Mynott

Abstract

Background—K88 positive enterotoxigenic *Escherichia coli* (K88$^+$ ETEC) is an important cause of diarrhoea in young piglets. K88$^+$ ETEC pathogenesis relies on attachment to specific glycoprotein receptors located on the intestinal mucosa. Proteolytic treatment of these receptors in vitro and in vivo prevents attachment of K88$^+$ ETEC to piglet small intestines and may be of clinical use to prevent K88$^+$ ETEC pathogenesis.

***Aims*—To determine whether bromelain, a proteolytic extract obtained from pineapple stems, would protect piglets against K88$^+$ ETEC diarrhoea and to confirm and extend earlier findings on the effects of bromelain on K88$^+$ ETEC receptors in vivo.**

***Methods*—Bromelain (0, 12.5, or 125 mg) was orally administered to just weaned piglets for 10 days. One day following commencement of bromelain treatment, piglets were challenged with K88$^+$ ETEC (5 $\times$ 10^{10} K88ac:0149) for seven days. Intestinal contents from unchallenged piglets were obtained via an intestinal fistula, and tested for their ability to bind K88$^+$ ETEC before and after bromelain treatment.**

***Results*—Both doses of bromelain were successful in reducing the incidence of K88$^+$ ETEC diarrhoea and protected piglets from life threatening disease. Bromelain treated pigs also had significantly increased weight gain compared with untreated pigs. Bromelain only temporarily inhibited K88$^+$ ETEC receptor activity, with receptor activity being regenerated 30 hours following treatment, consistent with the regeneration of new enterocytes.**

***Conclusion*—Results show that bromelain can temporarily inactivate ETEC receptors in vivo and protect against ETEC induced diarrhoea. Bromelain may therefore be an effective prophylaxis against ETEC infection.**

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Keywords: enterotoxigenic *Escherichia coli*; K88 ETEC; ETEC receptors; diarrhoea; bromelain

Victorian Institute of Animal Science, Attwood
D S Chandler

School of Agriculture, La Trobe University, Bundoora, Victoria, Australia
T L Mynott

Correspondence to:
Dr T L Mynott, Imperial College of Science, Technology, and Medicine, Department of Biochemistry, Exhibition Road, London SW7 2AZ, UK.

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Enterotoxigenic *Escherichia coli* that possess the K88 pilus (K88$^+$ ETEC) are commonly associated with diarrhoeal disease in young piglets.[1] Specific attachment of K88$^+$ ETEC to glycoprotein receptors[2 3] on the small intestinal mucosa is critical for colonisation and effective toxin delivery (reviewed in Gaastra and de Graaf[4]). K88$^+$ ETEC produce both heat labile (LT) and/or heat stable (STI and STII) enterotoxins. Some pigs are resistant to colonisation and disease caused by K88$^+$ ETEC[5] because their intestines lack functional K88 receptors. These pigs are referred to as being of a non-adhesive phenotype.[6] Piglets of the adhesive phenotype are susceptible to infection because they possess the appropriate intestinal receptors for K88$^+$ ETEC. A genetic basis exists for expression of the adhesive or non-adhesive phenotype.[5 7] In addition to genotype, physiological factors, particularly the level of intestinal proteolysis within the small intestine, influence the ability of ETEC to attach to intestine.[8–10]

Recently, we showed that oral administration of enteric protected bromelain, a proteolytic extract obtained from pineapple stems, prevented K88$^+$ ETEC attachment to piglet small intestine.[10] Bromelain treated piglets resembled those of the genetically determined, non-adhesive phenotype. Untreated pigs, in contrast, resembled piglets of the adhesive phenotype. Presumably bromelain treatment prevented ETEC recognition of intestines because of proteolytic cleavage of K88$^+$ ETEC receptors. As bromelain can prevent attachment of K88$^+$ ETEC to intestine, bromelain may be of clinical use to prevent diarrhoea.

In this study, we showed that bromelain reduces the incidence of K88$^+$ ETEC diarrhoea and protects piglets from life threatening disease. Bromelain treated pigs also had a significant increase in weight compared with untreated pigs. Intestinal contents, obtained via an intestinal fistula, were tested for their ability to bind K88$^+$ ETEC before and after bromelain treatment. Results show that bromelain only temporarily inhibited K88$^+$ ETEC receptor activity, with receptor activity being regenerated 30 hours following treatment, which is consistent with the regeneration of new enterocytes. The results extend our initial observations and confirm that bromelain can inactivate ETEC receptors in vivo and also show that bromelain protects against ETEC induced diarrhoea. Given the similarities in the mechanism of pathogenesis of ETEC strains that affect piglets and humans these data suggest that bromelain may be of clinical use to protect against human ETEC infections.

Methods

PIGLETS AND HOUSING

Approval for animal experiments was granted by the Victorian Institute of Animal Science

Animal Experimentation Ethics Committee. The study was designed to simulate the management and environmental variables of a commercial pig farm. Ninety three unweaned, male piglets (Large White × Landrace) aged 21 days were obtained from the University of Melbourne commercial pig farm (Mt Derrimut, Victoria, Australia). Earlier studies of 155 piglets had shown that the frequency of $K88^+$ ETEC non-adhesive phenotype in this herd is 0.097 (approximately 1 resistant pig in 10).[11]

Challenge studies were conducted on three groups of piglets (n=42, n=21, and n=30 respectively) on three separate occasions over a period of five months. On arrival at the animal housing facility, piglets were weighed and randomly assigned to three treatment groups. Piglets were immediately weaned and fed ad libitum a commercial starter diet for one week after weaning, followed by a grower diet (Barastoc, Australia). Each treatment group comprised seven replicates per treatment (four or five piglets per replicate). Treatment groups were housed in separate heated pens in the animal housing facility at the Victorian Institute of Animal Science.

PIGLET TREATMENT

Piglets were orally administered either 1 g (high dose, n=28) or 0.1 g (low dose, n=33) of enteric protected bromelain (Detach, Cortecs Ltd, Middlesex UK; 1 g of Detach contains 125 mg bromelain, EC 3.4.22.4). Bromelain treatment commenced one day prior to weaning and continued daily for 10 days. The bromelain preparation was suspended in water (5 ml) and administered by mouth using a plastic syringe to which a soft rubber tube (50 mm long) had been attached. Control animals received no medication (n=32) but were handled and restrained in a similar manner to those receiving medication.

BACTERIAL CHALLENGE

Previous attempts in our laboratory to reproduce $K88^+$ ETEC infection in weaned pigs by oral inoculation of bacteria had met with varied success (Chandler DS, Mynott TL, unpublished data; Tzipori SR, personal communication). To optimise the chance of successfully inducing disease, piglets were challenged with a mixed dose (5×10^{10} cells per dose) of 10 recently collected field isolates of $K88^+$ ETEC (all K88ac:0149). Bacteria were collected from pigs with diarrhoea on two commercial farms and from piglets that had been submitted to the laboratory for postmortem examination following death from post-weaning diarrhoea. The mixed dose of bacteria ensured that piglets were challenged with a combination of strains, each confirmed as producing LT, STI, or STII (confirmed by gene probe, RP Monckton, Bendigo Regional Veterinary Laboratory). Bacteria were administered at least 30 minutes following bromelain medication on the day of weaning and daily thereafter for seven days.

Stock cultures of individual strains were suspended in Trypticase soy broth (Oxoid) containing 15% (vol/vol) glycerol and stored in multiple aliquots at −80°C. A new aliquot of each strain was inoculated onto separate sheep blood agar (SBA; 5% vol/vol, Oxoid) plates and lawn cultures were grown overnight at 37°C. Cultures were harvested into sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2), pooled, and diluted to yield 5×10^{10} cells/5 ml dose. The bacterial concentration was confirmed by viable cell count on duplicate SBA plates after serial dilution in PBS. Prior to the inoculation of piglets, cultures were confirmed as bearing K88 pilus by slide agglutination against specific hyperimmune rabbit serum.

EFFECT OF ETEC CHALLENGE ON CLINICAL CONDITION

Piglets were weighed on the day before weaning, and 3, 7, 10, 15, and 21 days following weaning. Assessments of disease were made daily for 21 days after challenge by an observer blinded to the treatments. A disease score of 0 represented no signs of diarrhoea, lethargy, or dehydration; 1, faeces softer than normal, but with overall demeanour unaffected; 2, faeces liquid but with little or no evidence of lethargy or dehydration; 3, appearance of prolonged diarrhoea, with obvious dehydration and/or lethargy; and 4, diarrhoea with severe, life threatening dehydration and lethargy. Piglets were considered to be dehydrated if a pinch of skin at the base of the neck did not return to its original shape. Lethargic piglets were identified as those who were motionless in the pen or required stimuli to move. Piglets of condition 4 were removed from the study, killed by barbiturate overdose, and necropsy was performed. These piglets were recorded as deaths.

Disease scores were analysed in terms of the mean total disease score obtained per piglet and the mean number of scores greater than 2 recorded per piglet over the challenge period. Disease scores were subjected to analysis of variance (ANOVA) using Genstat V. The former assessment was intended to provide an overall indication of piglet health; the latter score was to provide an indication of the incidence of life threatening disease.

BACTERIOLOGY

To confirm that challenge bacteria passed into the bowel of piglets, rectal swabs were obtained from the first group of experimental pigs (n=42) at the same time as recording the disease score. *E coli* strains associated with post-weaning diarrhoea in Australia are predominantly haemolytic[12] (as were the challenge strains). Bacteria from rectal swabs were cultured on SBA agar and the presence of haemolytic *E coli* (Hly^+) colonies in microbiological cultures was confirmed. The presence of $K88^+$ ETEC was confirmed by slide agglutination with hyperimmune anti-K88 rabbit serum.

DETERMINATION OF K88 PHENOTYPE IN CHALLENGED PIGS

Three to four weeks after completion of the study, piglets were sent for slaughter at a commercial abattoir. At slaughter, intestines were removed, placed on ice, and transported to the laboratory. Intestines were processed within

two hours of death and the K88 phenotype of animals was determined by enzyme immunoassay (EIA) as previously described.[8 10 13] Briefly, K88$^+$ ETEC were immobilised to wells of a microtitre plate and incubated with piglet intestinal mucosa samples. Mucosal material bound to the bacteria was detected with antibody (rabbit IgG) raised against porcine intestine followed by urease conjugated goat antirabbit IgG (Sigma) and urea substrate. The period between completion of bromelain treatment and slaughter was adequate to allow regeneration of a new population of enterocytes in order to negate any potential influence of bromelain treatment on the immunoassay.[14]

DETERMINATION OF K88$^+$ ETEC RECEPTOR ACTIVITY IN BROMELAIN TREATED PIGS

To confirm that K88$^+$ ETEC receptor activity was inhibited by bromelain treatment, control animals aged 21 days (not receiving bacterial challenge, n=8) were investigated for receptor activity before and after treatment. Intestinal contents were used for assay of receptor activity as we have previously shown that small intestinal contents contain sufficient K88$^+$ ETEC receptors to identify the phenotype of pigs.[8] Samples of intestinal contents were obtained by means of an intestinal fistula fitted 100 cm proximal to the ileocaecal junction. Samples were collected via a syringe fitted to a soft Teflon tube (4 mm internal diameter) which was inserted approximately 100 mm along the intestine. Samples from five pigs were collected at least twice daily for two days prior to treatment (five samples per pig) and at approximately three hourly intervals for 24 hours following treatment (nine samples per pig). Immediately on collection, intestinal contents were diluted in a working dilution buffer as previously described,[10] but with the addition of trypsin inhibitor (0.1% wt/vol; soyabean, Type II-2, Sigma). The reason for the addition of trypsin inhibitor was to stabilise K88$^+$ ETEC receptor activity in stored samples. In studies in which receptor activity was monitored in the same piglet for several days, samples needed to be stored prior to conducting assays. We had noted previously that K88 receptor activity declined in samples stored for periods longer than two days (Chandler DS, Mynott TL, unpublished data) and we hypothesised that this loss of receptor activity was a result of autolytic breakdown of K88$^+$ ETEC receptors by endogenous trypsin. Inclusion of trypsin inhibitor in freshly assayed samples had no effect on K88$^+$ ETEC receptor activity (EIA activity (A_{540nm}): diluent alone, 0.75 (0.02); diluent + trypsin inhibitor, 0.70 (0.03)). In samples which had been stored for two days, there was a reduction in K88 receptor activity (diluent alone, 0.23 (0.04)), which was prevented by the addition of trypsin inhibitor (diluent + trypsin inhibitor, 0.65 (0.02)).

K88 receptor activity was also monitored more extensively in three piglets: at approximately 30 minute intervals, for three days. To minimise stress to the animals, caused by continuous handling, samples were obtained via a Teflon tube that was permanently fixed to the fistula. The tubing was connected to a peristaltic pump which operated at a rate which ensured a mild vacuum (flow rate 0.5 ml/min). To allow the piglets to move freely within their pen and to minimise tension on the fistula, the weight of the tube between the pig and the pump was balanced on a freely moving, counterbalanced line.

Samples were collected in diluent containing trypsin inhibitor (10×, 1 ml) and were maintained on ice throughout each collection period. Samples were collected every 10 minutes for approximately two hours following feeding, for a minimum of eight hours. Sample collection was automated by means of a fraction collector (Frac 100, Pharmacia) connected to the peristaltic pump. Three successive samples were pooled and standardised to contain equivalent amounts of intestinal contents by dilution. K88$^+$ ETEC receptor activity was then assayed on 100 µl of diluted sample as previously described.[10]

FITTING OF ILEAL FISTULAS

Unweaned piglets were obtained at 5–7 days of age and fed four times daily (100 ml/feed) with reconstituted full cream evaporated milk (Carnation). Feeding volumes increased to 500 ml/feed when piglets were 21 days of age (age when sampling commenced). Piglets were housed in separate pens and acclimatised to their surroundings for one week prior to surgery. Prior to sampling of intestinal contents, the surgical incisions were allowed to heal for one week. Piglets were killed by barbiturate overdose on completion of studies.

Piglets were anaesthetised (Halothane, Rhone Merieux, Australia) and fitted with intestinal fistulas by making an incision 40 mm long in the centre of the abdominal wall, 25 mm to the side of the midline. Fistulas were constructed from a stainless steel tube (9 mm outside diameter, 6 mm inside diameter; 50 mm in length) shaped into a "Y" shape and fitted with a removable screw cap lid.

Results

EFFECT OF BROMELAIN TREATMENT ON PIGLET CLINICAL CONDITION

Piglets were orally administered either 0, 0.1, or 1 g of enteric protected bromelain (equivalent to 0, 12.5, or 125 mg bromelain) for 10 days and challenged with K88$^+$ ETEC for seven days starting on day 2 of bromelain treatment. Mild diarrhoea was apparent (score 1) in 50% of all piglets one day following bacterial inoculation (fig 1). In earlier studies we noted that rechallenge with ETEC bacteria was necessary to produce more than transient diarrhoea in healthy, weaned piglets (Chandler DS, Mynott TL, unpublished data). Rechallenge with K88$^+$ ETEC in this study ensured diarrhoea (minimum of score 1) in 80% of untreated piglets by day 4. Diarrhoea was most severe in untreated piglets (100% of pigs attained at least score 2), five days after commencement of challenge. Diarrhoea (score 1 to 3) persisted in these pigs for three days after the final inoculation. At day 5 following challenge, only 40% of high dose and 50% of

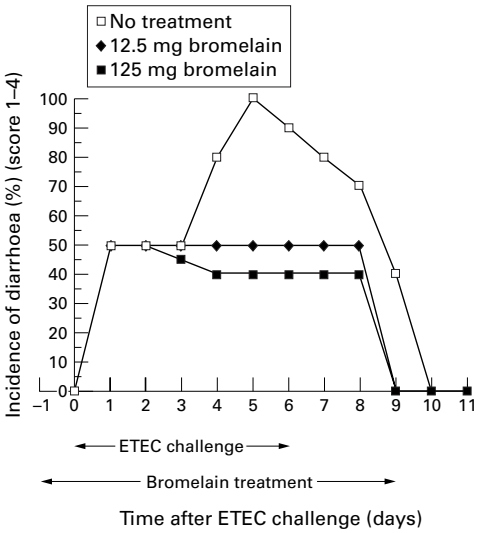

Figure 1 Incidence of diarrhoea (combined score 1 to 4) in piglets challenged with K88⁺ ETEC.

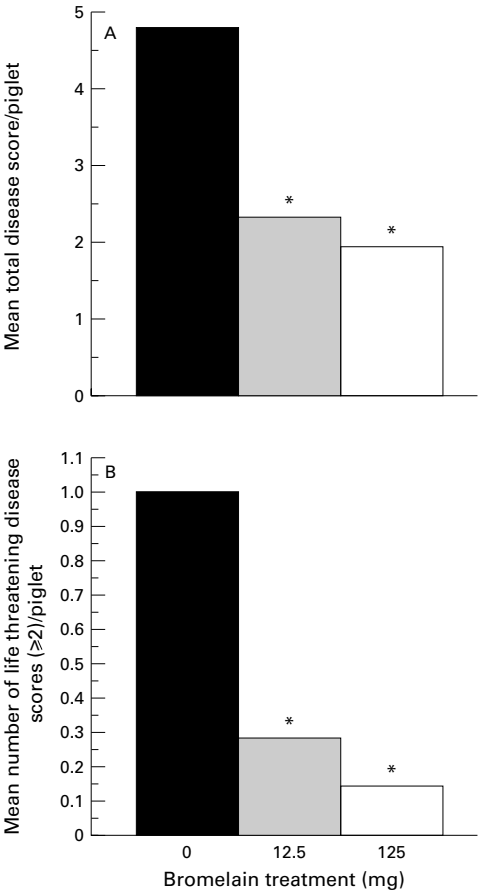

*Figure 2 Effect of bromelain treatment on piglets challenged with K88⁺ ETEC. (A) Mean total disease score. (B) Mean number of life threatening disease scores. *p<0.05.*

low dose treated pigs were affected with diarrhoea (score 1 to 2) (fig 1). An overall assessment of disease over the entire challenge period (score 1 to 4, combined) showed that piglets treated with bromelain had a significantly lower disease score than untreated piglets ($p<0.05$), indicating an overall increase in well being of the bromelain treated piglets (fig 2A). An assessment of disease scores at five

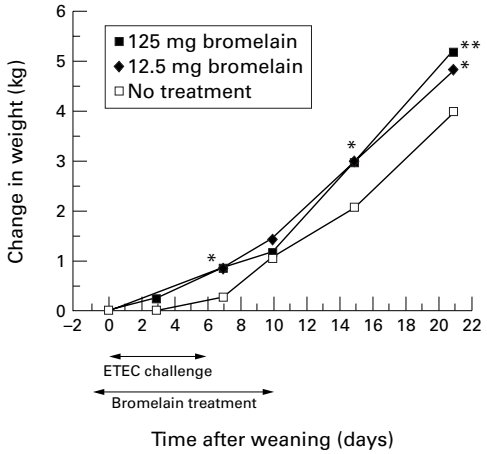

*Figure 3 Effect of bromelain treatment on weight gain of piglets challenged with K88⁺ ETEC. *p<0.05; **p<0.01.*

days, when diarrhoea was most severe (mean number of disease scores of 2 to 4), revealed that bromelain gave significant protection against life threatening disease (fig 2B). In the untreated piglets, a disease score of 2 or greater was made per piglet in comparison with only 0.27 and 0.14 (one pig in seven) in the low and high bromelain treated groups, respectively. Although piglets treated with the high dose of bromelain (125 mg) appeared to have a lower disease score than low dose treated pigs (12.5 mg), this difference was not statistically significant.

Five piglets died or were sacrificed because of poor health following bacterial challenge; three were in the untreated group and two in the low dose group. Piglet deaths were lower than expected, given the severity of the bacterial challenge. Post-weaning diarrhoea in piglets is a difficult disease to reproduce under experimental conditions (Chandler DS, Mynott TL, unpublished data; Tzipori SR, personal communication); however, the incidence of diarrhoea as indicated by disease scores, showed sufficient success at reproducing ETEC induced infection in this challenge model.

BACTERIOLOGY

Bacterial cultures of rectal swabs showed that all piglets excreted 90–100% Hly⁺ colonies, four to five days after commencement of bacterial challenge. A random sample of these colonies was confirmed to be K88⁺ by specific antisera. There was no difference in the excretion pattern of K88⁺ ETEC obtained between treatment groups. The presence of K88⁺ ETEC colonies in the rectum confirmed that the oral challenge of bacteria passed into the bowel of piglets.

EFFECT OF ETEC CHALLENGE ON PIGLET WEIGHT GAIN

The weight of piglets was monitored throughout the study as a quantitative indication of overall piglet health. Figure 3 shows the weight gain of piglets within each treatment group. Piglets treated with bromelain had a significantly larger weight gain than untreated pigs at 7, 15, and 21 days post-weaning ($p<0.05$,

Table 1 Incidence of K88⁺ ETEC non-adhesive phenotype

	Bromelain treatment		
	None	*Low dose (12.5 mg)*	*High dose (125 mg)*
No of piglets tested	23	27	30
Frequency of K88⁺ non-adhesive phenotype	0.17	0.07	0.10

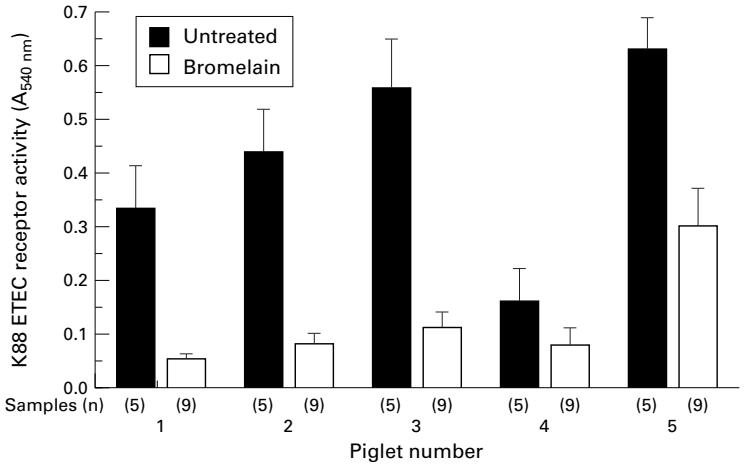

Figure 4 K88⁺ ETEC receptor activity of small intestinal contents of piglets before and after bromelain treatment. Bars represent the mean (SD) EIA activity ($A_{540\ nm}$) of intestinal samples taken from piglets before (n=5) and after (n=9) bromelain treatment.

ANOVA). There was no significant difference in weight between the two dose levels of bromelain. Exclusion of piglets from weight gain analysis because of death did not bias growth performance in favour of any treatment. The ratio of weight gains between untreated pigs, low dose, and high dose treated pigs in the three different study groups, revealed a similar normal:low:high weight gain ratio (data not shown).

DISTRIBUTION OF K88 PHENOTYPE IN CHALLENGED PIGLETS

A random sample of 80 pigs was assessed for K88 phenotype (table 1). A chance, increased distribution of K88⁺ ETEC non-adhesive (and disease resistant) pigs was found in the untreated pigs. This chance distribution towards the untreated piglets may have improved weight gains and disease status in favour of the control group. A similar frequency of non-adhesive phenotype was found in both the low and high treatment groups (table 1).

K88⁺ ETEC RECEPTOR ACTIVITY IN PIGLETS BEFORE AND AFTER BROMELAIN TREATMENT

To confirm that bromelain treatment inhibited K88⁺ ETEC receptor activity, eight unchallenged piglets were investigated for receptor activity before and after treatment. Figure 4 shows the K88⁺ ETEC receptor activity of five animals sampled. The K88⁺ ETEC receptor activity of piglet intestinal contents after treatment was significantly lower than before treatment (mean EIA activity of all piglets: before treatment, 0.42 (0.17); after treatment, 0.12 (0.10); p<0.009, Student's *t* test for paired observations), confirming our earlier results which showed that oral administration of bromelain inhibits K88⁺ ETEC receptor activity.[10]

K88⁺ ETEC receptor activity was also monitored more extensively in three piglets, at

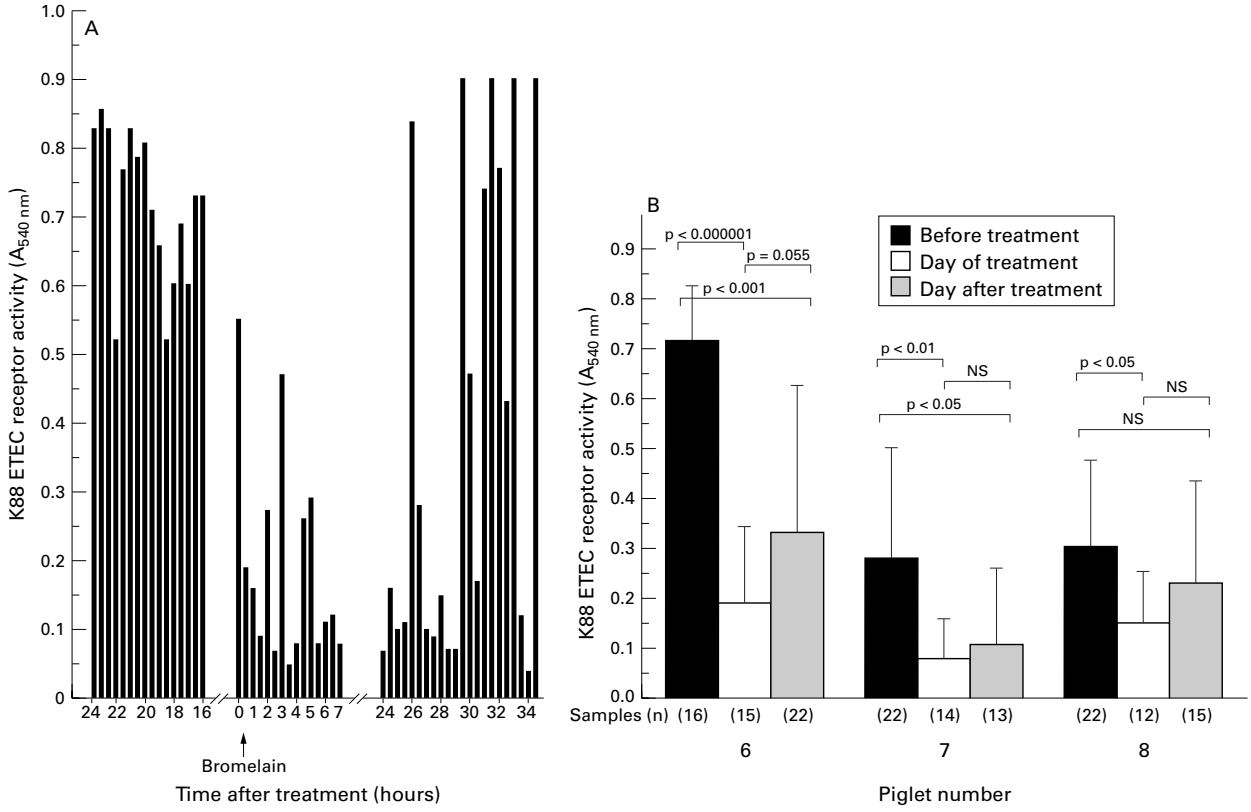

Figure 5 Change in K88⁺ ETEC receptor activity of small intestinal contents following bromelain treatment. (A) Results obtained from one piglet (no 6); receptor activity assayed at 30 minute intervals. (B) Mean receptor activity of intestinal contents for each day of sample collection. Numbers in parentheses indicate number of samples assayed. Significance determined by Student's t test for paired observations.

approximately 30 minute intervals, for three days. These studies were designed to determine the fluctuating levels of intestinal K88$^+$ ETEC receptors in vivo and to observe the regeneration of receptor activity after bromelain treatment. Figure 5A shows results obtained from one piglet. High receptor activity in intestinal contents was seen to be relatively constant during the pretreatment period. However, 30 minutes after bromelain treatment, K88$^+$ ETEC receptor levels were significantly reduced and remained low for the duration of the collection period of seven hours after treatment (mean EIA activity 0.72 (0.11) at 16 to 24 hours before treatment, versus 0.19 (0.15) at 30 minutes to seven hours after treatment; $p<0.000001$). Receptor levels were still low 24 hours following treatment (mean EIA activity 0.72 (0.11) at 16 to 24 hours before treatment, versus 0.38 (0.34) at 24 to 35 hours after treatment; $p<0.001$); they were, however, higher than the receptor levels immediately following treatment (mean EIA activity 0.72 (0.11) at 30 minutes to seven hours after treatment, versus 0.38 (0.34) at 24 to 35 hours after treatment; $p=0.055$). Receptor activity seemed to increase approximately 30 hours after bromelain treatment. Similar results were also obtained in the two other piglets studied (see fig 5B).

Discussion

Attachment of K88$^+$ ETEC to glycoprotein receptors on the small intestinal mucosa, is an important initial factor in the establishment of diarrhoeal disease. In vitro, protease treatment of human, porcine, and bovine small intestine inhibits receptor activity and attachment of ETEC strains which carry the CFA/I and CFA/II, K88 and K99 adhesins, respectively.[3 15 16] As ETEC receptors are sensitive to protease treatment, we hypothesised that one possible way of preventing ETEC diarrhoea would be to prevent attachment of bacteria by proteolytically modifying receptor attachment sites. Recently we confirmed that oral administration of bromelain, a proteolytic extract from pineapple stems, could inhibit ETEC receptor activity and K88$^+$ ETEC attachment to porcine small intestine.[10] In the present study, we also showed that oral administration of bromelain can protect against life threatening disease and improve the health status of piglets challenged with K88$^+$ ETEC. All untreated piglets challenged with K88$^+$ ETEC had diarrhoea (at least score 2) five days after challenge commenced. In comparison, only 40% of high dose bromelain treated pigs and 50% of low dose treated pigs had diarrhoea (score 1 to 2).

Bromelain medication was administered daily throughout a seven day bacterial challenge period. Repeated bromelain medication was to ensure that newly formed ETEC receptors, which would have been generated because of enterocyte turnover, were constantly exposed to bromelain. Studies investigating the regeneration of receptor activity, in which samples were taken from a fistula fitted in piglet small intestine, showed that K88 receptors do begin to regenerate approximately 30 hours after a single dose of bromelain. The regeneration rate of K88$^+$ ETEC receptor activity observed in this study is consistent with reports that the mammalian small intestinal epithelial cell population is replaced every one to three days.[17] Cells originating in villus crypts migrate and differentiate up the length of the villus to replace the epithelial cells lining the tips which have become extruded and desquamated in the lumen.

As receptor levels seem to be significantly reduced for a long period after bromelain treatment, it is quite feasible that less frequently administered doses of bromelain would be effective in preventing diarrhoea. Reduced doses would probably be effective in most situations in which continual high exposure to pathogens is unlikely. Indeed, we have shown that a single dose of bromelain is effective in preventing diarrhoea caused by CFA/I$^+$ ST$^+$LT$^+$ *E coli* (H10407) in the removable intestinal tie, adult rabbit diarrhoea model.[9] In further studies conducted on commercial pig farms we have also shown that a single oral dose of bromelain is effective in preventing diarrhoea in piglets (Chandler DS, Mynott TL, unpublished data). These data suggest that it is possible to prevent diarrhoea by administering bromelain prior to the predicted onset of bacterial challenge. Such treatment may be useful in the prevention of ETEC disease in piglets at weaning or travellers' diarrhoea in humans.

All piglets exhibited a similar pattern of excretion of K88$^+$ ETEC bacteria. This is despite the fact that bromelain treated pigs had less diarrhoea than untreated pigs. These data suggest that the mechanism of protection by bromelain is not bactericidal. The data also indicate that although the bacteria are present in sufficient numbers to induce disease (as indicated by diarrhoea in untreated pigs), they do not do so. We hypothesise that although bacteria are present, they are not able to attach to the small intestine and efficiently deliver their enterotoxins to target epithelial cells. The ability of bromelain to inhibit ETEC attachment ability is illustrated by enzyme immunoassays with which we assayed piglet intestinal contents for K88 ETEC receptor activity before and after bromelain treatment. Intestinal contents obtained via an intestinal fistula clearly exhibited significant reductions in K88$^+$ ETEC receptor activity following bromelain treatment. Use of this ileal fistula technique may assist further investigations of the modulation of bacterial receptors in vivo. From this study, it is clear that sufficient bacterial receptors exist in intestinal contents; they may be contained in the mucus or originate from enterocytes that have been sloughed into the lumen. The presence of receptors in intestinal contents would be particularly advantageous to the host if free receptor could capture ETEC, and the receptor-ETEC complex be flushed out of the lumen by intestinal peristalsis.[18]

Another possible explanation for the protective efficacy of bromelain in vivo, is that it may prevent diarrhoea by inhibiting the action of ETEC bacterial toxins. Recently Mynott *et al*[19]

showed in Ussing chamber studies that bromelain inhibits intestinal secretion induced by LT, STa, and *Vibrio cholera* cholera toxin as well as secretion by secretogogues that do not interact with a cell surface receptor, including theophylline (a phosphodiesterase inhibitor), calcium ionophore A23187 (releases intracellular Ca^{2+}), 8-bromoadenosine-3',5'-cyclic monophosphate, and 8-bromoguanosine-3',5'-cyclic monophosphate (cyclic nucleotide analogues).[19] Therefore bromelain, in addition to a direct proteolytic effect on mucosal ETEC receptors, has an antisecretory effect which is independent of an action on enterocyte receptors.

No adverse effects due to bromelain treatment were observed. An increase in weight of bromelain treated piglets supports no adverse effect of treatment on piglet health. The weight gain observed in bromelain treated pigs in this study is consistent with results obtained from field trials conducted on commercial pig farms. A study of 1107 pigs (552 untreated pigs, 555 bromelain treated) showed up to 60% reduction in piglet mortality and a 12% increase in weight in bromelain treated pigs.[20] Earlier studies with higher doses of bromelain also showed no adverse effect of treatment.[10]

Diarrhoeal diseases caused by ETEC remain an important health problem in children and young animals. Currently there is no effective way of preventing ETEC infection and several attempts to develop vaccines against ETEC infection are in progress. To be effective, new vaccines must be multivalent because of the number of different adhesins of differing antigenicity found on pathogenic ETEC. The data presented in this study represent a simple approach to prevent ETEC infection. We have shown that increased proteolytic activity in the small intestine reduces ETEC receptor activity, and hence resistance to ETEC colonisation and disease. The use of proteases could prevent disease caused by different ETEC bacteria that possess different adhesins and therefore provide broad spectrum protection.

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