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ORIGINAL ARTICLE

Fermented soya bean (tempe) extracts reduce adhesion of enterotoxigenic *Escherichia coli* to intestinal epithelial cells

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Keywords
anti-diarrhoeal effect, bioactivity, Caco-2, enterotoxigenic *Escherichia coli*, piglet brush borders cells, tempe.

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Abstract

Aims: This study aimed to investigate the effect of processed soya bean, during the successive stages of tempe fermentation and different fermentation times, on adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to intestinal brush border cells as well as Caco-2 intestinal epithelial cells; and to clarify the mechanism of action.

Methods and Results: Tempe was prepared at controlled laboratory scale using *Rhizopus microsporus* var. *microsporus* as the inoculum. Extracts of raw, soaked and cooked soya beans reduced ETEC adhesion to brush border cells by 40%. Tempe extracts reduced adhesion by 80% or more. ETEC adhesion to Caco-2 cells reduced by 50% in the presence of tempe extracts. ETEC K88 bacteria were found to interact with soya bean extracts, and this may contribute to the observed decrease of ETEC adhesion to intestinal epithelial cells.

Conclusions: Fermented soya beans (tempe) reduce the adhesion of ETEC to intestinal epithelial cells of pig and human origin. This reduced adhesion is caused by an interaction between ETEC K88 bacteria and soya bean compounds.

Significance and Impact of the Study: The results strengthen previous observations on the anti-diarrhoeal effect of tempe. This effect indicates that soya-derived compounds may reduce adhesion of ETEC to intestinal cells in pigs as well as in humans and prevent against diarrhoeal diseases.

Introduction

Diarrhoea is a major health problem worldwide both in human and in livestock populations, particularly during the weaning period of infants and farm animals such as piglets. Enterotoxigenic *Escherichia coli* (ETEC) is one of the major pathogens associated with mild and severe diarrhoea in children in the developing countries (Bhan 2000). ETEC strains are also an important and global cause of severe watery diarrhoea in the offspring of some animal species, such as newborn calves and suckling and weaned piglets (Nagy and Fekete 2005). ETEC strains adhere to, and colonize the intestinal mucosa and produce their enterotoxins, resulting in a decreased absorption of fluid and electrolytes from the intestinal lumen, ultimately leading to diarrhoea (Nataro and Kaper 1998).

Tempe is a traditional fermented food made from soaked and cooked soya beans inoculated with a mould, usually of the genus *Rhizopus*. After fermentation, the soya beans are bound together into a compact cake by a dense cottony mycelium. During the fermentation process, the levels of anti-nutritional constituents are decreased and the nutritional quality and digestibility of the fermented product are improved due to the enzymatic activity of the mould (Nout and Kiers 2005). The mould also contributes to the development of a desirable texture, taste and aroma of the product (Hachmeister and Fung 1993).

Tempe was reported to contain an antibacterial compound acting especially against some Gram-positive

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bacteria (Wang *et al.* 1969, 1972; Kobayasi *et al.* 1992; Kiers *et al.* 2002). Tempe also has been shown to inhibit ETEC infection in rabbits (Karyadi *et al.* 1990; Karmini *et al.* 1997). Research also indicated that in ETEC-challenged weaned piglets, diarrhoea was less severe on a tempe-based diet compared with a control diet containing toasted soya beans (Kiers *et al.* 2003). Paediatric research in Indonesia indicated that in infants, the recovery after acute bacterial diarrhoea was faster when tempe was consumed as an ingredient of the infant food formula (Karyadi and Lukito 1996, 2000; Soenarto *et al.* 1997). The reported beneficial effects of tempe in the control of diarrhoeal disease could be due to several factors, such as (i) decreased adhesion of bacteria to the intestinal epithelial cells because of interference of tempe compounds with bacterial and or epithelial cell surfaces, (ii) inhibition of bacterial toxin secretion or (iii) inhibition of growth of pathogenic ETEC by antibacterial compounds in tempe. As this mechanism still needs to be elucidated, the present study will specifically address aspects (i) and (iii).

Kiers *et al.* (2002) focused on ETEC adhesion to piglet brush border cells, as affected by the final product of soya bean fermentation (tempe). In this study, Caco-2 cells were used to study the adhesion of bacteria to intestinal epithelial cells. These Caco-2 cells can undergo spontaneous differentiation and achieve the characteristics of mature enterocytes, such as structural polarization, tight junctions, apical microvilli and enzyme expression (Chantret *et al.* 1988). Caco-2 cells have been widely used to study interactions of different bacteria (Wells *et al.* 1999; Lievin-Le Moal *et al.* 2002), including ETEC (Darfeuille-Michaud *et al.* 1990; Gaastra and Svennerholm 1996; Roselli *et al.* 2003) with host epithelial cells.

In the present study, adhesion inhibiting activity after the major processing stages (soaking, cooking and fermentation stages) of the tempe manufacture will be investigated, in order to verify whether the fermentation step is required to generate its bio-activity. In addition, the effect on the ETEC K88 adhesion to human intestinal epithelial cells (Caco-2) will be measured, offering an independent alternative to piglet brush border cells.

Materials and methods

Soya bean products

Dry-dehulled full-fat yellow-seeded soya beans (*Glycine max*) were soaked overnight in tap water at 30°C. In order to achieve an accelerated lactic acid fermentation during this soaking step, the soak water had been inoculated with naturally acidified soaking water ('backslop') (Nout *et al.* 1987). Next, the soya beans were rinsed with tap water and cooked in fresh tap water for 20 min at a bean : water ratio of 1 : 3. Subsequently, the beans were cooled and their surface dried at room temperature, and were spread out on mesh trays for about 1 h. For the fungal inoculation of the soya beans, a sporangiospore suspension was used, prepared by scraping off the sporangia from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU 573) grown on malt extract agar (CM59; Oxoid, Basingstoke, UK) for 7 days at 30°C, and suspending them in sterile distilled water with 0·85% NaCl and 0·1% peptone. After inoculation with the sporangiospore suspension (10 ml kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans), the beans (batches of 450 g) were packed into hard-plastic, perforated boxes ($205 \times 90 \times 45$ mm) and incubated for 48, 72, 96 and 120 h. Fermented soya beans, as well as raw, soaked and cooked soya beans, were freeze-dried and ground (Ultra Centrifugal Mill ZM 200; Retsch GmbH, Haan, Germany) passing through a 0·5-mm sieve and were stored at −20°C until further processing.

Freeze-dried products (20 g) were suspended and stirred for 1 h at room temperature in 200 ml of 35 mmol l^{-1} sodium phosphate buffer (pH 8·0), while the pH was adjusted with 2 mol l^{-1} NaOH every 30 min. The soluble extract was obtained by centrifugation (30 min, 25 000 ***g***, 20°C), followed by filtration of the supernatant through a folded paper filter (no. 311651, Schleicher & Schuell GmbH, Dassel, Germany), a second centrifugation step (30 min, 25 000 ***g***, 20°C), and freeze-drying of the supernatants. These were stored at −20°C and used as soya bean extracts in the experiments. Figure 1 outlines the preparation of the soya bean products studied.

ETEC growth in the presence of soya bean extracts

Growth of ETEC in media containing three concentrations of soya bean extracts was monitored by measurement of optical density. Enterotoxigenic *Escherichia coli* (O149:K91:$K88^{ac}$) strain ID1000 (ETEC K88; source: Animal Science Group, Lelystad, the Netherlands) was cultured overnight in brain heart infusion (BHI) broth (237500, Becton Dickinson, France) at 37°C. Cultures were centrifuged and re-suspended in BHI to a concentration of 10^6 CFU ml^{-1}. Soya bean extracts were dissolved at dry matter concentrations of 1, 2·5 or 10 g l^{-1} in BHI and 200 μl of these extracts were added to triplicate flat bottom wells of a sterile 96-well microplate (655161, Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Bacterial suspensions (50 μl) were added to the plate followed by incubation of the plate for 6 h at 37°C in a microplate reader. The optical density was monitored at a wavelength of 600 nm at intervals of 5 min with 5 s of shaking prior to each measurement

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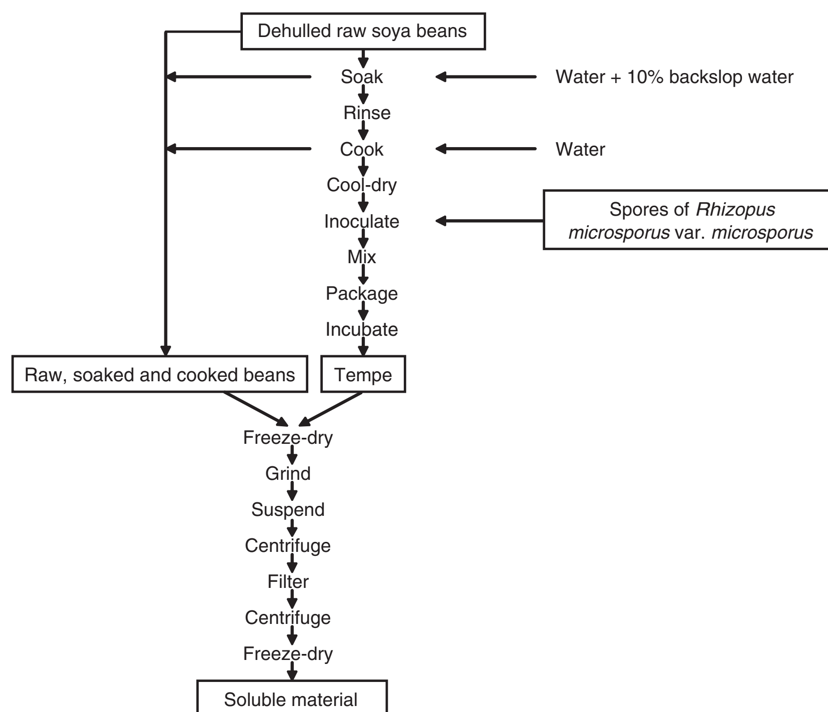

Figure 1 Flow diagram of the preparation of soya bean products and their extracts.

(SpectraMax Plus 384; Molecular Devices Ltd, Wokingham, UK).

Adhesion of ETEC to brush border cells

Brush border cells isolated from the jejunum of a K88-receptor positive, early weaned 6-week-old piglet were used according to Sellwood *et al.* (1975). The brush border cells were exposed to ETEC K88 (O149:K91:K88^{ac}) to confirm positive receptor status; brush borders that bound more than eight ETEC K88 (O149:K91:K88^{ac}) per brush border cell were recorded as K88-positive. In our experiments, we also used a nonadhering *E. coli* (O149:K91) strain ID1084 (source: Animal Science Group, Lelystad, the Netherlands) as a negative control.

The ETEC K88-positive (ID 1000) and K88-negative strain (ID1084) were grown overnight at 37°C in BHI. The cultures were centrifuged (3000 ***g***, 10 min) and washed twice with phosphate-buffered saline (PBS) (NaCl 136·89 mmol l^{-1}, KCl 2·68 mmol l^{-1}, Na_2HPO_4 8·1 mmol l^{-1}, KH_2PO_4 2·79 mmol l^{-1}, pH 7·2), followed by suspending the pellets in PBS, to a optical density of 0·75 correlating with 10^9 CFU ml^{-1}.

Freeze-dried soya bean extracts (10 mg) were dissolved in 1 ml PBS, mixed in a head-over-tail rotator for 1 h and centrifuged (10 000 ***g***, 10 min) and further diluted to 2·5 and 1 g l^{-1}. Diluted extracts (50 μl) were mixed with 50 μl of ETEC K88 suspension and 50 μl of brush border cell suspensions, and the mixture was incubated at room temperature with continuous gentle shaking (100 min^{-1}) in a plate shaker for 1 h. The number of bacterial cells adhering to 12 individual brush border cells was determined by phase contrast microscopy (magnification 1000×). The proportion of adhesion was calculated as the average number of ETEC K88 per brush border cell, relatively to the adhesion with a PBS control.

The effect of pre-incubation of two out of the three components, namely brush border cells, ETEC and tempe extract (tempe fermented for 72 h, extract containing 1 g l^{-1} dry matter) was tested by pre-incubation during 30 min prior to addition of the third component; adhesion to brush border cells was then measured after an additional 30 min of incubation. In some cases, a washing with PBS was performed to remove excess tempe extract before the third component was added. Table 1 summarizes the combinations tested.

Adhesion of ETEC to Caco-2 epithelial cells

The human intestinal Caco-2 cell line (American Type Culture Collection, Rockville, MD, USA) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with addition of 25 mmol l^{-1} Hepes and 4·5 g l^{-1} glucose supplemented with 10% heat inactivated (30 min 56°C) foetal bovine serum, 1% nonessential amino acids, L-glutamine (2 mmol l^{-1}) and gentamycin (50 μg ml^{-1}) in an atmosphere of 5% CO_2/95% air at 37°C. All cell culture reagents were obtained from Invitrogen (Breda, the

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Table 1 Adhesion of ETEC to brush border cells, as affected by composition and timing of reaction mixture

Treatment No.*	Sample mixture†		Washing‡	Addition (after 30 min)§	Adhesion (%)¶
1 (Pos. control)	BB	ETEC+	None	PBS	100 ± 2·7[a]
2 (Neg. control)	BB	ETEC−	None	PBS	3·4 ± 1·6[b]
3	BB	ETEC+	None	TE	87·9 ± 4·4[a]
4	BB	TE	None	ETEC+	4·1 ± 2·1[b]
5	BB	TE	PBS (once)	ETEC+	61·6 ± 6·7[c]
6	ETEC+	TE	None	BB	7·4 ± 2·3[b]
7	ETEC+	TE	PBS (once)	BB	2·7 ± 1·9[b]
8	ETEC+	TE	PBS (twice)	BB	2·0 ± 1·1[b]
9	ETEC+	PBS	PBS (twice)	BB	72·3 ± 2·3[c]

*Treatment number corresponds to the sample mixture mentioned in columns 2 and 3.

†Initial mix of two components: BB: 50 μl brush border cells, ETEC+: 50 μl ETEC strain ID 1000, ETEC−: 50 μl ETEC strain ID1084, TE: 50 μl Tempe extract 1 g l^{-1} of 72 h fermented tempe, PBS: 50 μl Phosphate buffered saline.

‡Washing by centrifugation of the sample mixture followed by suspension in PBS.

§Addition of the third component.

¶Adhesion expressed as % of the positive control (treatment 1) without tempe addition ± SEM. Significant differences are indicated by different superscript alphabets.

Netherlands). Cells were sub-cultured after they had reached 70–90% confluence in plastic tissue culture flasks with a growth area of 75 cm^2 (Corning B.V, Schiphol-Rijk, the Netherlands). For bacterial adhesion experiments, cells were seeded in 12-well tissue culture plates (Corning B.V, Schiphol-Rijk, the Netherlands) at a concentration of 5×10^5 cells ml^{-1} culture medium. In each well, 1 ml of the cell suspension was pipetted and the plates were left in the incubator for 3 weeks to allow differentiation. The medium was refreshed three times per week. Cells at passages 35–50 were used for experiments.

Freeze-dried soya bean extracts (12·5 mg) were suspended in 5 ml tissue culture medium (TCM) without serum and gentamicin. Extracts were centrifuged (3000 ***g***, 10 min) and filtered through 0·22 μm filters (FP30/0·2CA-S, Schleicher & Schuell GmbH, Dassel, Germany). Cytotoxicity of tempe extracts to Caco-2 cells was tested using the lactate dehydrogenase (LDH) leakage test (Mitchell *et al.* 1980).

The ETEC K88 strain was cultured overnight in BHI at 37°C. The culture was washed twice and re-suspended in TCM, to a density of about 10^9 CFU ml^{-1}. Caco-2 cells were washed once with PBS (pH 7·4) and 1 ml of soya bean extract was added to triplicate wells, followed by 50 μl of bacterial suspension. To allow ETEC adhesion, the plates were incubated for 1 h at 37°C. Nonadhered bacteria were removed by three washes with 1 ml of PBS. Caco-2 cells were then lysed with 1 ml of 1% triton-X-100 (T8787, Sigma) and viable ETEC K88 were quantified by plating appropriate serial dilutions of lysates on tryptone soy agar (TSA) (CM 0131, Oxoid, Basingstoke, UK). To quantify bacterial adhesion to Caco-2 cells, each combination of soya bean extract and Caco-2 cells was tested in three independent replications performed on different days and in triplicate tissue culture wells. The adhesion was plotted relatively to a control, without soya bean extract, which was defined as 100%.

ETEC adhesion to soya bean extracts

Bacterial adhesion to soya bean extracts was measured according to Becker *et al.* (2007). The freeze-dried soya bean extracts were suspended in PBS (pH 7·2) to concentrations of 1, 2·5 and 10 g l^{-1} and centrifuged (460 ***g***, 5 min, 21°C). The flat-bottomed wells of high-binding polystyrene microtitration plates (Microlon F plate 655092; Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands) were coated with 300 μl of the supernatant and incubated overnight at 4°C. Noncoated wells were included as negative controls in each plate. Subsequently, plates were washed with 300 μl PBS buffer to remove nonbound material, and the plates were blocked by adding 300 μl of 10 g l^{-1} bovine serum albumin (BSA) A7906; Sigma-Aldrich, Zwijndrecht, the Netherlands) in PBS per well, and incubation at 4°C for 1 h. Subsequently, the plates were washed twice with 300 μl of PBS to remove excess BSA. ETEC was grown overnight, washed and suspended in PBS to an optical density of 0·02 (600 nm, path length 1 cm) corresponding to about 10^7 CFU ml^{-1}, and 300 μl of this bacteria suspension was added into the wells. Bacteria were allowed to adhere to the coated soya bean extracts at room temperature for 30 min. Then, the wells were washed three times with 300 μl of PBS to remove nonadhered bacteria, and 250 μl BHI broth was added to each well. The microtitre plate was placed in a microplate reader (SpectraMax plus 384; Molecular Devices Ltd, Wokingham, UK), where it was incubated at 37°C and shaken at medium intensity for 5 s prior to every reading. The OD was determined at a wavelength of 600 nm in intervals of 5 min. All readings were performed in triplicate and the experiment was replicated once. The optical density measurement was used as a tool to compare numbers of adhered bacteria to the coated soya bean extracts. A correlation between the time-dependent detection of the growth by optical density measurement and the initial cell density of adhered bacteria in plates is demonstrated in Becker *et al.* (2007). The OD was determined at a wavelength of 600 nm in intervals of 5 min. All readings were performed in triplicate and the experiment was replicated.

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Statistical analyses

Significance of adhesion of ETEC to Caco-2 cells was determined by the Student's *t*-test assuming equal variances. The significance of the other experiments was evaluated by comparing means using one or two-way ANOVA, followed by the Bonferonni posttest. Results were expressed as mean ± SEM and differences were considered significant when $P < 0{\cdot}05$. Statistics were performed with Graphpad PRISM ver 4.03 for Windows (Graphpad Software, San Diego, CA, USA).

Results

Effect of soya bean extracts on the growth of ETEC

Figure 2 shows the effect of raw, soaked, cooked and fermented soya bean extracts added to BHI in three concentrations, on the growth of ETEC K88. Optical densities of cultures, growing in soya bean extract medium, after 4 h incubation (mid-logarithmic phase) were compared with the control culture in BHI. None of the extracts inhibited the growth of ETEC K88. Instead, almost all fermented and nonfermented soya bean extracts showed a small but significant increase in optical density compared with the control. These results showed no antibacterial effect of the soya bean products to ETEC K88 within the tested concentrations. There were also no significant concentration effects of the soya bean extracts within a group of soya bean extracts on the growth of ETEC K88.

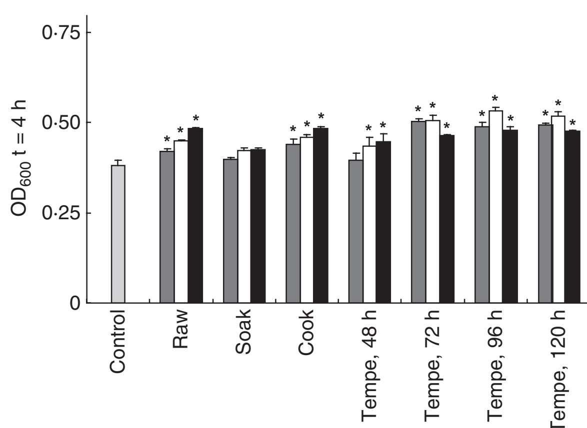

Figure 2 Effect of soya bean extracts on the optical density of ETEC K88 in BHI. Figure shows OD_{600} after 4 h of growth of ETEC K88 in BHI (control: light grey bar) and BHI with added soya bean extracts 1 g l^{-1} (grey bar), 2·5 g l^{-1} (white bar) and 10 g l^{-1} (black bar) concentrations. Bars with asterisk (*) are significantly different from control. Error bars represent SEM of three independent experiments.

Adhesion of ETEC K88 to brush border cells

Incubation of brush border cells with the ETEC K88 strain resulted in adhesion of, on an average, approximately 10 bacterial cells to one brush border cell; we defined this value as 100%. In contrast, the *E. coli* K88 negative strain showed an adhesion of one bacterial cell per brush border or less (5%). The effect of the different soya bean extracts on ETEC K88 adhesion to brush border cells is presented in Fig. 3.

In the presence of 10 g l^{-1} soya bean extracts, a complete inhibition of the adhesion was observed with all extracts. At the level of 2·5 g l^{-1}, raw, soaked and cooked soya bean extracts resulted in almost 50% inhibition of adhesion compared with the control, whereas the tempe extracts reduced adhesion to levels similar to the negative control. At the level of 1 g l^{-1}, there was only a significant inhibition of ETEC adhesion observed with the fermented soya bean (tempe) extracts. Longer fermentation times (48–120 h) did not significantly affect the inhibition activity.

During the microscopic observation, it was observed that after the incubation, some aggregation of ETEC K88 cells took place, particularly in the presence of the two highest concentrations of tempe extracts. This clumping of ETEC K88 did not occur at the surface of brush border cells.

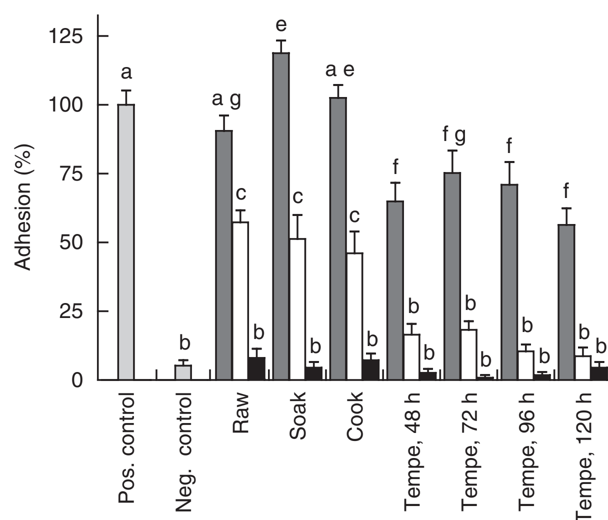

Figure 3 Adhesion of ETEC K88 to piglet intestinal brush border cells. Adhesion is expressed relative to a control (100%) without added soya bean extract (light grey bar). Soya bean extracts were added as 1 g l^{-1} (grey bar), 2·5 g l^{-1} (white bar) and 10 g l^{-1} (black bar) concentrations. The negative control represents adhesion of an ETEC-negative nonadhering strain. Error bars represent the SEM of 12 brush border cell counts. Means without a common letter differ significantly.

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Adhesion of ETEC K88 to Caco-2 epithelial cells

In the absence of soya bean extracts, approximately 2×10^6 CFU ml^{-1} of ETEC cells adhered to Caco-2 cells; this value was defined as 100% adhesion. In Fig. 4, the effect of 2·5 g l^{-1} of soya bean extracts on the adhesion of ETEC K88 to Caco-2 cells is shown. We estimated that this soya concentration is of an order of magnitude that can be encountered in the intestine after consumption of a serving of tempe. The used concentration (2·5 g l^{-1}) did not cause any damage to the Caco-2 cells themselves as tested with a LDH-leakage test (data not shown).

Extracts of raw, soaked and cooked soya beans showed a slight but significant increase of ETEC K88 adhesion, compared with the control. Treatment with the tempe extracts resulted in a significant inhibition of adhesion of almost 50% compared with the control. Longer fermentation times did not show additional effect on the inhibitory activity on the adhesion, only 120-h fermented tempe showed an even lower adherence percentage.

ETEC K88 adhesion to soya bean extracts

Becker *et al.* (2007) demonstrated a correlation between the time-dependent detection of the growth by optical density measurement, and the initial cell density in plates. Higher initial numbers, for instance of adhering cells, result in shorter detection times of bacterial growth upon incubation. Figure 5 shows the optical density curves of ETEC K88 adhered to a plate coated with 2·5 g l^{-1} soya bean extract as a representative of the curves obtained. Based on this correlation and the observations in Fig. 5, significantly more ETEC K88 was bound to all of the soya bean extracts, compared with the control. Figure 6 shows two independent replications of optical density (OD) curves for 2·5 g l^{-1} soya bean extract concentration, compared at OD = 0·4 level. This level was selected as a criterion for its location in the linear part of all the optical density curves (see Fig. 5) and it corresponds with about 10^8 CFU ml^{-1}. A comparison of the mean times to achieve OD = 0·4 reveals the same trend in both the replications, although the significant ($P < 0{\cdot}05$) difference of about 20 min occurred due to variations in initial bacterial load (namely, $2{\cdot}5 \times 10^7$ CFU ml^{-1} for replication 1 and $1{\cdot}9 \times 10^7$ CFU ml^{-1} for replication 2) and timing of the experiments.

The wells coated with the extracts of tempe fermented for 72, 96 or 120 h all showed similar growth curves in both the replications, reaching a mean OD = 0·4 after 3·2 ± 0·01 h (mean ± SEM) compared with the control 4·8 ± 0·13 h (mean ± SEM) for replication 1. Tempe extracts gave similar adhesion as the soaked bean extracts. The adherence of ETEC to raw and cooked soya bean extracts is significantly lower than to tempe extracts.

Other soya bean concentrations (1 and 10 g l^{-1}) did not show significant different results and are not shown.

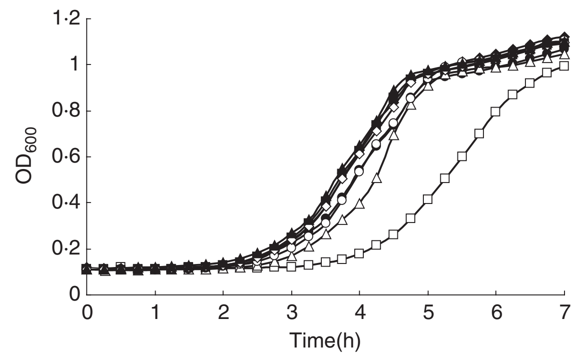

Figure 5 Growth curves of adhered ETEC K88 to soya bean extracts figure shows the growth of adhered bacteria on plates coated with 2·5 g l^{-1} of soya bean extract of one replication in triplicate. Lines with different symbols represent the control (open square), raw (closed circle), soaked (closed diamond) and cooked (open triangle) soya beans and soya beans fermented for 48 h (open circle), 72 h (closed square), 96 h (open diamond) and 120 h (closed triangle). Optical density was measured every 5 min, but for clarity only 15 min time point are shown in this figure. Error bars of the three measurements are not visible in the figure since they were smaller than the symbols shown.

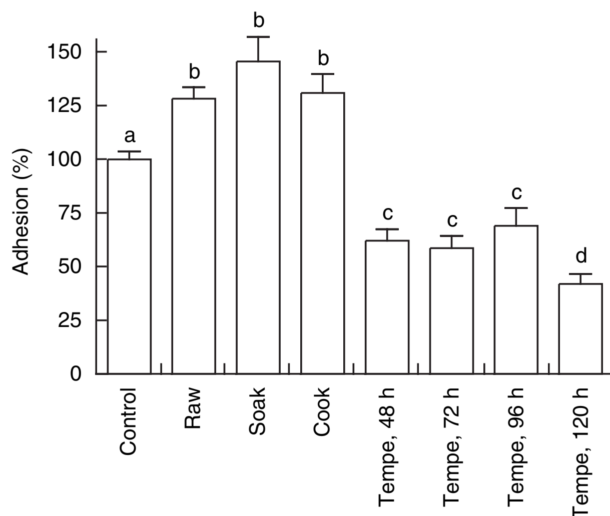

Figure 4 Adhesion of ETEC K88 to Caco-2 cells, as influenced by soya bean extracts. Bacterial adhesion is expressed relative to a control (100%) without added soya bean extract. Soya bean extracts were added at 2·5 g l^{-1}. Error bars represent SEM values of three independent experiments carried out in triplicate (n = 9). Means without a common letter differ significantly.

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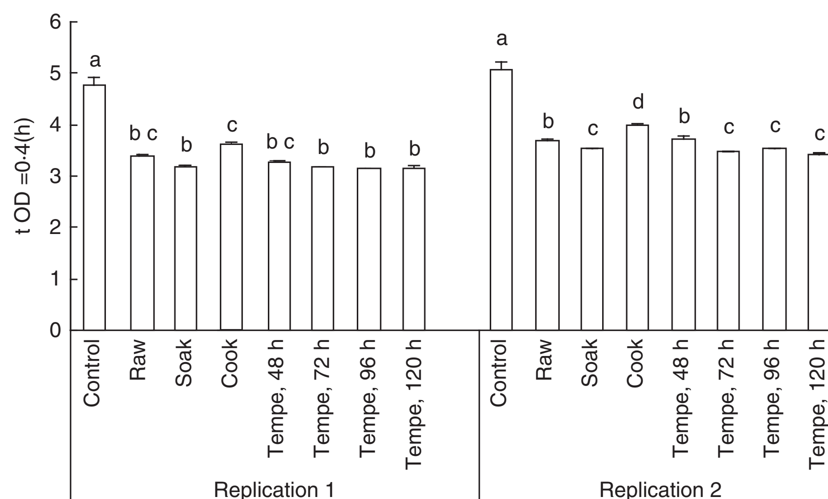

Figure 6 Adhesion of ETEC K88 to soya bean extracts. Bacterial adhesion is expressed as the mean time (h) to reach OD = 0·4 at 600 nm. For clarity, only results of 2·5 g l^{-1} soya bean extracts are presented. Error bars represent SEM of three measurements of the extracts and nine measurements of the control. Left side of the figure shows replication 1 and the right side replication 2. Means without a common letter differ significantly.

Interaction between brush border cells, ETEC and tempe extracts

Table 1 shows a range of mixtures made in a different order. Treatments 1 and 2 represent the positive and negative controls, respectively. Treatment 6 shows that pre-incubation of ETEC with tempe extract results in strongly reduced adhesion, comparable to the negative control. Removing tempe extract by washing (treatments 7 and 8) did not change this low adhesion. Treatment 4 shows that also the presence of tempe extract prior to the addition of ETEC blocks the adhesion of ETEC to brush border cells. After incubation of brush border cells with ETEC for 30 min followed by addition of tempe extract (treatment 3), the bacteria already adhered to the brush border cells and adhesion was not reduced. ETEC bacteria showed a strong interaction with tempe extracts. When brush border cells were exposed to tempe extract and washed before ETEC addition (treatment 5), the adhesion was lower than the positive control but much higher than without washing of the tempe extracts. The tempe extracts appear to interact specifically with the ETEC bacteria.

Discussion

We examined the effect of soya bean extracts on the adherence of ETEC K88 to intestinal epithelial cells. Whereas all soya bean extracts protected intestinal piglet brush border cells against ETEC K88 adhesion to some extent, the extracts of fermented soya beans (tempe) had the highest inhibitory effect on ETEC K88 adhesion. The tempe extracts also were the only extracts that protected human intestinal epithelial cells against ETEC K88 adhesion.

It has been suggested that a protective effect by tempe extracts is achieved through its antibacterial activity (Wang *et al.* 1969, 1972; Kobayasi *et al.* 1992; Kiers *et al.* 2002). These studies demonstrated some antibacterial activity against Gram-positive bacteria such as *Bacillus* spp., but no antibacterial activity was reported against Gram-negative bacteria. In the present study, none of the soya bean extracts inhibited the growth of ETEC K88 as shown in Fig. 2. We therefore conclude that the inhibition of adhesion by soya bean extracts cannot have been caused by antibacterial activity on ETEC K88.

ETEC K88 have been reported to adhere to porcine brush border cells (Jones and Rutter 1972; Jin and Zhao 2000; Kiers *et al.* 2002) as well as to human Caco-2 cells (Sugita-Konishi *et al.* 2002; Roselli *et al.* 2003). Attachment to the mucosal surfaces is the first step in the pathogenesis of this bacterium, thus the inhibition of ETEC adhesion is crucial in preventing infection. The brush border cell adhesion inhibition assay quantifies the actual binding of the K88-positive ETEC to isolated piglet brush border cells. It should be realized that in these *in vitro* experiments not all conditions as those present in the intestine such as the presence of acids and hydrolytic enzymes can be reproduced.

The intrinsic micro-organisms (i.e. originating from intestinal microbiota) had been removed during the preparation and purification of the brush border cells as was confirmed by their absence in microscopical observation. Consequently, possibilities of interference of intrinsic microbiota with ETEC were not included in this study. However, *in vivo* studies (Kiers *et al.* 2003) have shown that anti-diarrhoeal effects are significant *in vivo*, implying that intrinsic microbiota has no nullifying effect. Inhibition of ETEC K88 adhesion to brush border cells was reported previously for several tempe extracts (Kiers *et al.*

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2002). In the present study, all processing stages of the tempe process were considered for their inhibitory effect on adhesion to brush border cells. Although not as effective as tempe, the extracts of raw, soaked and cooked soya beans also inhibited ETEC K88 adhesion, and for all soya bean extracts, higher inhibitory activity was observed at higher extract concentrations. The extract concentrations tested (1, 2·5 and 10 g l^{-1}) were physiologically relevant, considering a serving size of 100 g of tempe of which at least 30% of dry matter is solubilized by digestion; in the intestinal tract this would be diluted to concentrations of at least 10 g l^{-1} (Kiers *et al.* 2000). We also confirmed that this concentration did not cause any artefacts in our experiments by undue damages to the Caco-2, or brush border cells.

The *in vivo* protective effect of tempe in piglets (Kiers *et al.* 2003) implies that its bio-activity is not affected by digestive enzymes. We therefore focused on the mechanism of action, using undigested tempe extracts.

An important observation that can be made is that the response of both types of intestinal cells is not identical. Adherence of ETEC K88 to Caco-2 intestinal epithelial cells was inhibited by tempe extracts only, whereas adhesion to brush border cells also seems to be affected by other soya bean extracts. We should keep in mind that since both cell types originate from different species as well as different locations in the intestinal tract, they may have different interactions with tempe extracts and ETEC bacteria. In addition, the experimental conditions were not identical: brush borders cells were suspended in a buffer and single cells were observed, while Caco-2 cells grow in a layer that was only exposed to tempe extracts at the apical side of the cells. Nevertheless, both cell types revealed a high anti-adhesion bioactivity of the tempe extracts.

Longer tempe fermentation times did not result in higher inhibitory activities in the tempe extracts, neither in the brush border cells nor in the Caco-2 cells, so we expect that the bioactivity of the fermented soya bean extracts is released within the first 48 h of fermentation.

Our data show that ETEC K88 adheres to wells coated with soya bean extracts. The bioactivity of the soya bean extracts may be due to some kind of adhesion or interaction between soya components and the bacterial cells. This phenomenon was verified in the brush border adhesion assay shown in Table 1, which indicates that incubation of ETEC with tempe extracts leads to a coating or other surfacial change on the cell wall of the ETEC bacteria that cannot be undone by washing. The soya bean extracts could possibly form a layer coating the bacterial surface, or its specific fimbriae binding sites, so that they cannot adhere anymore to the epithelial cells, but instead aggregate into clumps of bacterial cells. Clumps of bacteria were indeed observed by microscopical examination.

In conclusion, soya bean extracts can protect against the adhesion of ETEC K88 to pig intestinal brush border cells *in vitro*. The fermented soya bean tempe extracts also protect Caco-2 epithelial cells against ETEC K88 adhesion. This effect is not a result of an antibacterial activity, but of an interaction between the soya bean extracts and the bacteria, resulting in a loss of adhesion capability of the ETEC to the intestinal cells.

Further study is required to elucidate the nature of the bioactive component in fermented soya bean extracts.

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