Effect of processed and fermented soyabean on net absorption in enterotoxigenic Escherichia coli-infected piglet small intestine

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Infectious diarrhoea is a major problem in both children and piglets. Infection of enterotoxigenic Escherichia coli (ETEC) results in fluid secretion and electrolyte losses in the small intestine. In the present study the effect of processed and fermented soyabean products on net absorption during ETEC infection was investigated. Soyabean was processed into an autoclaved, a cooked and a mould-fermented (tempeh) product. The soyabean products were pre-digested and the effect of the products on net absorption in the small intestine of piglets was studied. Pairs of small-intestinal segments, one non-infected and the other ETEC-infected, were perfused simultaneously with the different products during 8 h. Net absorption of fluid, DM, Na, chloride, K and total solutes was determined. Net fluid absorption was highest for cooked soyabean followed by autoclaved soyabean and tempeh as a result of the osmolality of these products. In ETEC-infected segments, cooked soyabean and tempeh showed minor fluid losses (27 (SE 23) and 43 (SE 20) µl/cm², respectively) compared with the saline control (260 (SE 23) µl/cm²). Tempeh resulted in a high uptake of solutes. Processed soyabean products, particularly cooked soyabean and tempeh, are beneficial in maintaining fluid balance during ETEC infection. Additionally, tempeh showed high DM and total solute absorption. Therefore, particularly, tempeh may be beneficial in the case of post-weaning diarrhoea in piglets and possibly in children as well.

Soyabean: Enterotoxigenic Escherichia coli: Absorption: Pigs: Diarrhoea

The incidence of diarrhoea due to enteric pathogen infections is particularly high in less-developed countries and in individuals travelling to these regions. Diarrhoeal diseases claim nearly two million lives annually among children less than 5 years of age in areas of the world with poor hygiene (World Health Organization, 1999). Enterotoxigenic Escherichia coli (ETEC) is one of the main pathogens associated with severe diarrhoea (Bhan, 2000). ETEC secretes heat-labile and/or heat-stable enterotoxins resulting in net fluid loss and, ultimately, diarrhoea. Problems with acute diarrhoea also frequently occur in pig husbandry. In piglets ETEC is the main aetiological agent in both pre- and post-weaning diarrhoea (Hampson, 1994).

Tempeh is a traditional fermented food made from soaked and cooked soyabean inoculated with a mould, usually of the genus Rhizopus (Nout & Kiers, 2005). After fermentation has occurred, the soyabean are bound together into a compact cake by dense cottony mycelium. An important function of the mould in the fermentation process is the synthesis of enzymes that hydrolyse soyabean constituents and contribute to the development of a desirable texture, flavour and aroma of the product. Enzymic hydrolysis also decreases or eliminates anti-nutritional factors and the nutritional quality is improved (Nout & Kiers, 2005).

Tempeh contains antibacterial substances against gram-positive bacteria (Wang et al. 1969, 1972; Kobayasi et al. 1992), and in vitro tempeh extracts are able to inhibit adhesion of ETEC to piglet small-intestinal brush border membranes (Kiers et al. 2002). Rabbits infected with ETEC and fed tempeh for 4 weeks showed reduced diarrhoea compared with rabbits fed diets without tempeh (Karmini et al. 1997), and in ETEC-challenged weaned piglets severity of diarrhoea was less on a diet with tempeh compared with a control diet containing toasted soyabean (Kiers et al. 2003). Beneficial effects for tempeh have been reported in diarrhoea management in Indonesian children (Karyadi & Lukito, 1996, 2000). Because of its protective effects during infection and its improved digestibility and nutrient availability, tempeh may be beneficial in the case of (post-weaning) diarrhoea and accelerating the recovery of young animals and young

Abbreviations: ETEC, enterotoxigenic Escherichia coli; SA, autoclaved soyabean; SC, cooked soyabean; SR, raw soyabean; ST, fermented soyabean.

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children, who are most at risk for ETEC diarrhoea and malnutrition.

Against the background of reported beneficial effects of tempeh in the control of diarrhoeal disease, in the present study the effect of processed and mould-fermented soyabean products on net absorption in non-infected and ETEC-infected piglet small intestine was investigated.

Materials and methods
Soyabean products
All products were produced from the same batch of soyabean (Fig. 1). Dehulled yellow-seeded soyabean (Glycine max) were soaked overnight in tap water using accelerated acidification (Nout et al. 1987). Subsequently, the beans were washed with tap water and cooked in fresh tap water at a 1:3 ratio for 20 min, cooled and superficially dried at room temperature. Sporangiospore suspension was obtained by scraping off the sporangia from pure slant cultures of Rhizopus microsporus var. microsporus LU 573 grown on malt extract agar (CM 59; Oxoid, Basingstoke, Hants, UK) for 7 d at 30°C and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone. After inoculation of the cooked soyabean with the sporangiospore suspension (1%, v/w) the beans (450 g) were packed into hard plastic perforated boxes with the sporangiospore suspension (1%, v/w) the beans (0.1% peptone. After inoculation of the cooked soyabean with the sporangiospore suspension (1%, v/w) the beans (450 g) were packed into hard plastic perforated boxes

Autoclaved soyabean (SA) was prepared by autoclaving ground raw soyabean for 30 min at 121°C and was subsequently pre-digested as was done for raw, cooked and fermented soyabean (Fig. 1). After pre-digestion the slurries were diluted using distilled water to approximately 6-5% DM and kept at 4°C until use as perfusion products the next day.

Net intestinal absorption
All procedures involving animal handling and testing were reviewed and approved by the Animal Care and Ethics Committee of the Animal Sciences Group (Lelystad, The Netherlands).

Fig. 1. Processing of soyabean, SR, raw soyabean; SA, autoclaved soyabean; SC, cooked soyabean; ST, fermented soyabean (tempeh).
Intestinal absorption of processed soybeans

Dolethal injection of 200 mg sodium pentobarbital/kg body weight responding drainage bottles (outflow). The piglets were killed by product remaining in the segments was blown out into the corresponding drainage bottles placed at the same level as the piglet’s abdomen. At the end of the experiment the product remaining in the segments was blown out into the corresponding drainage bottles (outflow). The piglets were killed by injection of 200 mg sodium pentobarbital/kg body weight (Dolethal®; Vetoquinol BV, ’s-Hertogenbosch, The Netherlands). The segments were cut from the mesenterium and the length was measured. Samples of 1 cm² were cut from the segment and put into physiological salt solution. Decimal dilution series were made and appropriate dilutions were spread on heart infusion agar (Difco™; BD Diagnostics, Franklin Lakes, NJ, USA) supplemented with 5% of defibrinated sheep blood. Plates were incubated for 18–24 h at 37 °C and haemolytic colonies were counted.

DM content of the products and outflows was determined by drying samples for 24 h at 80 °C in triplicate. Na, K and chloride concentrations of products and outflows were determined using an Electrolyte 4+ analyser (Noval Biomedical, Waltham, MA, USA). Osmolality was determined using a cryoscopic osmometer (Osmomat; Gonotec GmbH, Berlin, Germany).

Net fluid, DM, Na, K and chloride and total solute absorption were calculated from the difference between the volume and concentration of inflow and outflow divided by the surface area (length × circumference) of each segment.

Statistics

Results of non-infected and ETEC-infected segments perfused with the same product were compared using the Student’s paired t test, whereas comparisons between different perfusion products were done with ANOVA. All statistics were performed with GraphPad Prism version 4.00 for Windows (2004; GraphPad Software, San Diego, CA, USA). Net absorption of fluid, DM, Na, K, chloride and total solutes are presented as mean values with their standard errors.

Results

Characteristics of the pre-digested processed and fermented soyabean products are shown in Table 1. SR and SA products were very similar. K content in SR and SA was much higher compared with SC and ST. Na and chloride content were highest for ST and lowest for SC. Osmolality was lowest for SC and almost double that value in ST.

Net fluid absorption was highest for SC followed by SA and ST, whereas SR showed the lowest fluid absorption (Table 2). Perfusion of SR resulted in significantly lower net fluid absorption values compared with SA despite equal product characteristics (Table 1). An inverse linear relationship was found between net fluid absorption and osmolality of the three pre-digested processed soyabean products (SC, SA and ST) both for the non-infected (fluid absorption = 844–1·27 × osmolality; r² 0·99) and the ETEC-infected (fluid absorption = 808–1·33 × osmolality; r² 0·93) situation.

ETEC infection resulted in a decrease in net fluid absorption of 260 (SE 23) µl cm⁻² for saline. Reduction in average net fluid absorption was much less pronounced in soyabean products (Table 2). For SC and ST, net fluid absorption in ETEC-infected segments was not significantly lower compared with the non-infected segments. All soyabean products appeared to protect to a certain extent against fluid loss upon ETEC infection (Fig. 2). Mean fluid loss was considerably smaller for SC and ST when compared with SR and SA.

Net DM absorption was higher from soyabean products compared with saline and was significantly higher for processed soyabean compared with SR (Table 2). Although ST showed higher net DM uptake compared with SA and SC, these differences were not significantly different. Net DM absorption was not significantly different between non-infected and ETEC-infected segments for all processed soyabean products, but was for saline and SR.

Perfusion of SR resulted in net secretion of Na in both non-infected and ETEC-infected segments (Table 2). Na uptake was similar for SA, SC and ST in non-infected segments and was significantly reduced in ETEC-infected segments, although the reduction for ST was less severe. In addition, chloride and K absorption was significantly reduced as a result of the ETEC-infection, except for K absorption in the segments perfused with SA.

Perfusion of saline resulted in high net total solute absorption (Fig. 3). The sum of the net absorption of Na, chloride and K almost equalled the net total solute absorption for saline. SR showed net secretion of solutes in non-infected as well as ETEC-infected segments. Of all soyabean products ST showed highest net total solute absorption. It is clearly

![Table 1. Characteristics of pre-digested raw (SR), autoclaved (SA), cooked (SC) and fermented (tempeh; ST) soyabean products](https://www.cambridge.org/core/terms, Copyright © 2006 by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)
shown that for all tested products, except for ST, a net secretion of solutes other than Na, chloride and K is observed (Fig. 3). Apparently, tempeh was the only product showing significant absorption of solutes other than Na, chloride and K.

Bacteriological analysis of the mucosa from ETEC-infected segments showed high numbers of ETEC for all soyabean products (Table 3). Segments perfused with saline showed significantly lower ETEC numbers.

**Discussion**

Tempeh is a traditional fermented food made from soaked and cooked soybeans inoculated with a mould. Considerable leaching of soluble DM occurs (Kiers et al. 2000), which explains the low K content of the cooked and fermented soybean products. This leaching may also account for the rather low osmolality of SC. Osmolality is increased in ST as a result of breakdown of macromolecules during fermentation and the addition of excess HCl and NaHCO₃ during the predigestion to adjust pH for the different enzymic degradation steps (Kiers et al. 2000).

High DM uptake during perfusion with tempeh might reflect an improved nutrient availability after fermentation as suggested before (Kiers et al. 2000). It was probably not the result of differences in osmolality and Na concentration between cooked soybean and tempeh, because low osmolality does not affect the absorption rates of N and carbohydrate from nutrient solutions (Pfeiffer et al. 1998). Furthermore, the Na concentration of enteral diets does not influence the absorption of macronutrients and of total energy in minipigs (Ehrlein et al. 1999). When total net solute absorption was corrected for Na, chloride and K, only tempeh showed a positive balance, probably reflecting the uptake of easily accessible nutrients and/or minerals (Macfarlane et al. 1990; Mital & Garg, 1990; Kiers et al. 2000).

The negative correlation between the osmolality of the processed soybean products and net fluid absorption was shown earlier for oral rehydration solutions in rats (Hunt et al. 1991) and piglets (Kiers et al. 2001) and for oral rehydration solutions in which the glucose content had partially been

**Table 2.** Average net absorption of fluid, dry matter, sodium, chloride and potassium after perfusion of saline and pre-digested raw (SR), autoclaved (SA), cooked (SC) and fermented (tempeh; ST) soyabean products in piglet small-intestinal segments (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>Fluid (μl/cm²)</th>
<th>DM (mg/cm²)</th>
<th>Na (mmol/cm²)</th>
<th>Chloride (mmol/cm²)</th>
<th>K (mmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-infected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>469 ± 33</td>
<td>209 ± 42</td>
<td>1.5 ± 0.8</td>
<td>7.6 ± 0.8</td>
<td>16.0 ± 1.5</td>
</tr>
<tr>
<td>SR</td>
<td>340 ± 11</td>
<td>22 ± 17</td>
<td>1.3 ± 1.0</td>
<td>12.8 ± 2.3</td>
<td>11.5 ± 0.9</td>
</tr>
<tr>
<td>SA</td>
<td>581 ± 47</td>
<td>47 ± 14</td>
<td>1.7 ± 1.2</td>
<td>21.8 ± 2.3</td>
<td>16.6 ± 1.5</td>
</tr>
<tr>
<td>SC</td>
<td>646 ± 52</td>
<td>617 ± 50</td>
<td>1.5 ± 1.0</td>
<td>33.8 ± 2.0</td>
<td>25.6 ± 2.5</td>
</tr>
<tr>
<td>ST</td>
<td>465 ± 52</td>
<td>421 ± 42</td>
<td>0.8 ± 0.5</td>
<td>15.8 ± 1.5</td>
<td>10.8 ± 2.5</td>
</tr>
<tr>
<td><strong>ETEC-infected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>665 ± 74</td>
<td>74 ± 10</td>
<td>10 ± 2.4</td>
<td>27 ± 2.4</td>
<td>32 ± 3.5</td>
</tr>
<tr>
<td>SR</td>
<td>510 ± 74</td>
<td>14 ± 3.0</td>
<td>1.4 ± 0.4</td>
<td>28 ± 2.0</td>
<td>11 ± 1.5</td>
</tr>
<tr>
<td>SA</td>
<td>380 ± 47</td>
<td>13 ± 1.5</td>
<td>1.3 ± 1.0</td>
<td>12.8 ± 2.3</td>
<td>16.6 ± 1.5</td>
</tr>
<tr>
<td>SC</td>
<td>240 ± 47</td>
<td>10 ± 3.0</td>
<td>1.4 ± 0.4</td>
<td>27 ± 2.4</td>
<td>32 ± 3.5</td>
</tr>
<tr>
<td>ST</td>
<td>381 ± 52</td>
<td>10 ± 3.0</td>
<td>1.4 ± 0.4</td>
<td>27 ± 2.4</td>
<td>32 ± 3.5</td>
</tr>
</tbody>
</table>

Mean value was significantly different from that of the non-infected segments: *P < 0.05, **P < 0.01, ***P < 0.001.

Mean values within a column with unlike superscript letters were significantly different (P < 0.05).
replaced by amino acids or food supplements in rats (Pillai et al. 1994a, b).

The difference in net fluid absorption between non-infected and ETEC-infected segments was higher for saline compared with the different soyabean products. The observed different protective effects of the soyabean products were not caused by their different osmolalities, since osmolality of a perfusion solution does not affect the degree of reduction in net fluid absorption due to ETEC infection (Kiers et al. 2001). Especially cooked soyabean and tempeh minimised the reduction in net fluid absorption in ETEC-infected segments.

Various modes of action may be involved in diminishing the fluid loss upon ETEC infection by soyabean products. First, compounds in the soyabean products may interfere with attachment of ETEC to the enterocytes, as we showed for tempeh and pig brush borders in vitro before (Kiers et al. 2002). Second, the tempeh may still have proteolytic activity (proteases produced during fermentation) in the intestine that might inactivate ETEC receptors and consequently protect against ETEC-induced diarrhoea (Mynott et al. 1996, 1997; Chandler & Mynott, 1998). However, since high numbers of ETEC were detected after sampling the intestinal wall after perfusion, probably no conclusive interference with the adhesion of ETEC occurred during perfusion with the soyabean products. Third, the presence of insoluble compounds such as fibres may have contributed by affecting viscosity and/or transit time (Go et al. 1994). However, soyabean contains only small amounts of viscous fibre (Ehrlein & Stockmann, 1998) and also the viscosity of processed and fermented soyabean products is low (Osundahunsi & Aworh, 2002). Fourth, soluble fibres have been shown earlier to enhance intestinal water and electrolyte absorption in normal and secreting rat small intestine (Rabbani et al. 1991; Wingertzahn et al. 1999; Turvill et al. 2000). Finally, the protective effect could have resulted from inhibition of intestinal secretion by interaction with the enterotoxin as was shown for factors from rain-tree (Hor et al. 1995), or by inhibition of chloride channels as was shown for low-molecular-weight factors from rice (Macleod et al. 1995; Mathews et al. 1999).

The environment induced by the processed soyabean products and/or the presence of specific compounds and/or (in)soluble fibres of the processed soyabean products may explain the protective effect of these soyabean products. Further research is required to uncover the mode of action by which especially soyabean tempeh and cooked soyabean protect against ETEC-induced fluid losses in piglets and possibly in children.

### Table 3. Number of enterotoxigenic Escherichia coli (ETEC) attached to the infected segmental wall of piglet intestine after perfusion with saline and pre-digested raw (SR), autoclaved (SA), cooked (SC) and fermented (tempeh; ST) soyabean products (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Product</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6.55a</td>
<td>0.34</td>
</tr>
<tr>
<td>SR</td>
<td>8.25b</td>
<td>0.18</td>
</tr>
<tr>
<td>SA</td>
<td>8.62a</td>
<td>0.22</td>
</tr>
<tr>
<td>SC</td>
<td>8.90a</td>
<td>0.19</td>
</tr>
<tr>
<td>ST</td>
<td>8.73b</td>
<td>0.23</td>
</tr>
</tbody>
</table>

cfu, Colony-forming units.

a,b Mean values with unlike superscript letters were significantly different (P < 0.05).
Acknowledgements

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