

A high molecular weight soluble fraction of tempeh protects against fluid losses in *Escherichia coli*-infected piglet small intestine

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Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhoea in children and piglets. Infection of ETEC results in fluid secretion and electrolyte losses in the small intestine. In this study the effects of tempeh, a traditional fungal fermented soyabean product, on fluid losses induced by ETEC infection in piglets was investigated. Pairs of ETEC-infected and non-infected small intestinal segments of piglets were perfused simultaneously for 8 h with pre-digested tempeh, its supernatant and saline as an internal control. In saline perfused segments, ETEC infection reduced net fluid absorption by more than 500 $\mu\text{l}/\text{cm}^2$, whereas this reduction was significantly less for pre-digested tempeh and its supernatant (75 and 282 $\mu\text{l}/\text{cm}^2$, respectively). The supernatant of pre-digested tempeh was also compared with its permeate and retentate fractions. These fractions were created by ultra-filtration and contained respectively low and high molecular weight (>5 kDa) compounds. Again ETEC infection caused a significant reduction of net fluid absorption when perfused with saline (386 $\mu\text{l}/\text{cm}^2$) and also with the permeate fraction (300 $\mu\text{l}/\text{cm}^2$), but much less with the supernatant and the retentate fraction (125 and 140 $\mu\text{l}/\text{cm}^2$, respectively). The reduction in net fluid absorption upon ETEC infection when perfused with supernatant of either undigested or pre-digested tempeh was not different. Therefore from this study it can be concluded that a high molecular weight soluble fraction of tempeh is able to protect against fluid losses induced by ETEC, suggesting that this could play a potential role in controlling ETEC-induced diarrhoea.

Soyabean: Tempeh: ETEC: Absorption: Pig: Diarrhoea

One of the major pathogenic bacteria associated with acute diarrhoea is enterotoxigenic *Escherichia coli* (ETEC)^{1,2}. Diarrhoea caused by ETEC is a persisting health problem in children and young animals^{2–4}. Treatment options for acute gastroenteritis mainly rely on oral rehydration solution, although the volume, frequency or duration of diarrhoea are not reduced using conventional oral rehydration solution^{2,5}.

Tempeh is a traditional fermented food made from soaked dehulled and cooked whole soyabeans inoculated with a mould, usually of the genus *Rhizopus*⁶. An important function of the mould in the fermentation process is the synthesis of biomass and enzymes that hydrolyse soyabean constituents and contribute to the development of a desirable texture, flavour and aroma of the product. The process also inactivates or eliminates certain anti-nutritional factors and the enzymatic degradation improves the nutritional quality⁶.

Tempeh has been reported to contain an antibacterial substance^{7–9} and *in vitro* tempeh extracts are able to inhibit adhesion of ETEC to piglet small intestinal brush border membranes¹⁰. Rabbits infected with ETEC and fed tempeh showed reduced diarrhoea compared with rabbits fed diets without tempeh¹¹. Perfusion of small intestinal segments of piglets

with pre-digested tempeh strongly reduced ETEC-induced intestinal fluid losses¹² and in ETEC-challenged weaned piglets the severity of diarrhoea was reduced by a diet containing tempeh compared with a control diet containing toasted soyabeans¹³. In malnourished children tempeh was reported to be beneficial in terms of duration of diarrhoea episodes and rehabilitation period when supplemented to their diet^{14–16}.

The present study was undertaken to investigate whether the reduction of ETEC-induced intestinal fluid loss in piglets by tempeh can be attributed to insoluble or to water-soluble tempeh fractions of either low or high molecular mass.

Material and methods

Tempeh

Dehulled yellow-seeded soyabeans (*Glycine max*) were soaked overnight for about 16 h in tap-water while undergoing accelerated lactic acid fermentation using naturally acidified soaking water as an inoculum¹⁷. Subsequently, the beans were washed with tap-water and cooked (100°C) in fresh tap-water at a ratio of 1:3 for 20 min, and cooled to room

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*.

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temperature within 20 min by evaporation of adhering water by spreading them on perforated trays. 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 (Oxoid, CM 59) 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 soyabeans with the sporangiospore suspension (1 %, v/w), the beans (450 g) were packed in hard-PVC, perforated boxes (205 × 90 × 45 mm) and incubated at 30°C for 72 h. The resulting fresh tempeh was cut into 1 cm dice and dehydrated for 6 h at 60°C, ground using a 1.0 mm screen and stored at -20°C until use.

Tempeh was pre-digested as described earlier¹⁸. It was suspended in distilled water (5 g/30 ml) and incubated while stirring with 2 ml α -amylase solution consisting of 125 000 units/l α -amylase (A-1031; Sigma Chemical Co., St Louis, MO, USA), 1.5 g/l NaCl, 1.5 g/l K₂HPO₄, 0.5 g/l Na₂CO₃ (pH 7.0) for 30 min at 37°C. Next, the pH was adjusted to 4.0 using 5 M-HCl and the suspensions were incubated with 8 ml stomach-medium (0.1 g/l lipase (*Rhizopus* F-AP15; Amano Pharmaceuticals, Nagoyy, Japan), 0.125 g/l pepsin (P-6887; Sigma), 3.1 g/l NaCl, 1.1 g/l KCl, 0.6 g/l Na₂CO₃, 0.11 g/l CaCl₂, pH 4.0) for 1 h at 37°C. The pH was then adjusted to 6.0 using solid NaHCO₃. Finally, 10 ml of a 2 % pancreatic solution (20.0 g/l pancreatin (P-1750; Sigma), 5.0 g/l bile (B-3883; Sigma), 5.0 g/l NaCl, 0.68 g/l KH₂PO₄, 0.3 g/l Na₂HPO₄, 0.84 g/l NaHCO₃) was added and the suspensions were incubated for 30 min at 37°C. After pre-digestion the slurry was diluted using distilled water to 6.5 % DM.

Part of the suspension was centrifuged at 3000 g for 15 min at 4°C. The supernatant was filtered through a filter aid (Celite® 545, 22 140; Fluka, Buchs, Switzerland) to remove residual coarse particles and finally filtered through a 0.22 μ m filter (Steritop SCGPT05RE; Millipore, Billerica, MA, USA).

Supernatant of pre-digested tempeh was fractionated into permeate and retentate using ultra-filtration through a spiral wound membrane with molecular weight cut-off of 5 kDa (S2K328; Koch, Wilmington, MA, USA) against distilled water. In order to obtain fractions having the same volume as that of the original supernatant, permeate and retentate volumes were adjusted using rotary vacuum evaporation at 40°C.

Analysis

DM content of pre-digested supernatant, permeate and retentate was determined by drying aliquots until constant weight and nitrogen content was measured using a NA2100 nitrogen analyser (Interscience, Breda, The Netherlands). After centrifugation (10 min, 1300 g), sodium, potassium and chloride concentrations were determined using an Electrolyte 4 + analyser (Nova Biomedical, Waltham, MA, USA) and osmolality was determined using a cryoscopic osmometer (Osmomat; Gonotec, Berlin, Germany).

Gel permeation chromatography was performed on a LC-10Ai HPLC (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands) equipped with a Superdex Peptide column (17-5003-01; Pharmacia Biotech, Piscataway, NJ, USA) and elution at 30°C with 0.1 % (v/v) trifluoroacetic acid and 30 % (v/v) acetonitrile at 0.5 ml/min. The eluate was monitored using a UV detector at 200 nm. Calibration was

performed using proteins and peptides ranging from 200 to 7000 Da.

High-performance size-exclusion chromatography was performed on a SP8800 HPLC (Spectra Physics, Mountain View, CA, USA) equipped with three columns (each 300 × 75 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-Rad Labs, Hercules, CA, USA) in combination with a TSK guard column (40 × 6 mm) and elution at 30°C with 0.2 M-NaNO₃ at 0.8 ml/min. The eluate was monitored using a refractive index detector. Calibration was performed using dextrans ranging from 180 Da to 500 kDa.

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.

Piglets (crossbred Yorkshire × (Large White × Landrace)) were weaned at 3 weeks of age. About 2 weeks after weaning biopsies from the duodenal mucosa were taken using a fiberoptic (Olympus GIF XP10; Olympus, Hamburg, Germany) and receptor status was determined¹⁹. Sixteen piglets that expressed the receptor (K88/F4) involved in binding of the ETEC strain were used in the experiments 3 weeks after weaning.

The small intestinal segment perfusion test was carried out essentially as described before²⁰. Up to ten segments, with a cranial inflow and a caudal outflow tube and 20 cm long, were situated between 35 and 65 % of the total length of the small intestine.

At 15 min before the perfusion started, the odd-numbered segments were injected with 5 ml ETEC (5 × 10⁹ colony forming units, O149:K91:K88^{ac}, producing LT and STb) and the even-numbered segments with 5 ml PBS. In each piglet, pairs of segments (a non-infected and an adjacent ETEC-infected) were perfused with either saline (supplemented with 0.1 % glucose and 0.1 % casamino acids and serving as an internal control to determine the maximum response to the infection) or any of the tempeh products, using a Latin-square design. Each segment was perfused with 64 ml product over 8 h, by injecting 2 ml product every 15 min.

Three consecutive experiments were carried out. In experiment 1 the total pre-digested tempeh, its supernatant, saline (and another not relevant soyabean product) were tested in four piglets in a 4 × 4 Latin-square design. In experiment 2 saline, the supernatant of the pre-digested tempeh, the permeate and the retentate obtained after ultra-filtration were tested in eight piglets in a replicated 4 × 4 Latin-square design. In experiment 3 with four piglets saline, the supernatant of pre-digested tempeh, the supernatant of undigested tempeh (and another not relevant soyabean product) were tested in a 4 × 4 Latin-square design.

At the end of the small intestinal segment perfusion test the product remaining in the segments was blown out into the drainage bottles. The piglets were killed by injection of sodium pentobarbital (200 mg/kg body weight) and the segments were cut from the mesenterium and their length was measured.

Net fluid, sodium, chloride and solute absorption were calculated from the difference between the volume and

concentration of inflow and outflow divided by the surface area (length \times circumference) of each segment. Reduction in net fluid absorption upon ETEC infection was determined by subtracting net fluid absorption in ETEC-infected segments from net fluid absorption in non-infected segments perfused with the same perfusion fluid.

Statistics

Results of non-infected and ETEC-infected segments perfused with the same product were compared using the Student's paired *t* test. The effect of the perfusion fluid on net fluid absorption upon ETEC infection was analysed with ANOVA for a Latin-square design with piglet, pair of segment and treatment as model effects. Results on net fluid absorption and osmolality in experiment 2 were analysed using linear regression analysis. All statistics were performed with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Net absorption of fluid, DM, sodium, chloride and solutes are presented as means and their standard errors.

Results

Pre-digested tempeh and its supernatant

In experiment 1 ETEC infection in saline-perfused segments resulted in a decrease in net fluid absorption of more than $500 \mu\text{l}/\text{cm}^2$ compared with non-infected segments (Fig. 1). The reduction in net fluid absorption upon ETEC infection was significantly less when segments were perfused either with pre-digested tempeh or its supernatant. The difference in reduction in net fluid absorption upon ETEC infection between pre-digested tempeh ($75 \mu\text{l}/\text{cm}^2$; 14% compared with saline) and its supernatant ($282 \mu\text{l}/\text{cm}^2$; 53% compared with saline) was substantial but not significantly different.

Tempeh supernatant, permeate and retentate

Ultra-filtration of the supernatant of pre-digested tempeh resulted in a permeate fraction containing only low molecular weight compounds and a retentate fraction containing all compounds $>5 \text{ kDa}$ and some residual low molecular weight

compounds (Fig. 2). Similar chromatograms were observed for gel permeation chromatography protein/peptide analysis and areas under the curve showed that around 50% of the total DM in supernatant of pre-digested tempeh consisted of proteinaceous or protein-derived compounds (nitrogen $\times 6.25$) (Table 1). After ultra-filtration, the permeate contained the majority (approximately 65%) of the initial nitrogen. The osmolality of the retentate was the lowest and about a third of the permeate and a fourth of the supernatant.

In experiment 2 there was an inverse linear relationship between osmolality of the perfused fluids and net fluid absorption in non-infected segments (Fig. 3), with the net fluid absorption of retentate being the highest. Upon ETEC infection a significant decrease in net fluid absorption was observed for saline and the permeate (386 (SEM 69) and 300 (SEM 62) $\mu\text{l}/\text{cm}^2$, respectively). The decrease in net fluid absorption upon ETEC infection and perfusion with either supernatant or retentate was not significant, being 125 (SEM 37) and 140 (SEM 42) $\mu\text{l}/\text{cm}^2$, respectively. ETEC infection resulted in a decrease in net sodium and chloride absorption (Table 2). The reduction in sodium and chloride absorption was highest when perfused with saline and permeate and less when perfused with supernatant and retentate.

Pre-digested and undigested tempeh supernatant

Pre-digestion of tempeh did not result in liberation of soluble carbohydrate polymers $>8.5 \text{ kDa}$, whereas numerous low molecular weight compounds were formed (Fig. 4). Pre-digestion resulted in a marked increase in nitrogen levels (in low molecular weight compounds) and osmolality (Table 3).

The difference in net fluid absorption between non-infected and ETEC-infected segments in experiment 3 amounted to almost $400 \mu\text{l}/\text{cm}^2$ for perfusion with saline. Reduction in net fluid absorption upon ETEC infection was not different for undigested and for pre-digested tempeh supernatant (211 (SEM 72) and 214 (SEM 83) $\mu\text{l}/\text{cm}^2$, respectively) and represented a reduction of 54% compared with saline.

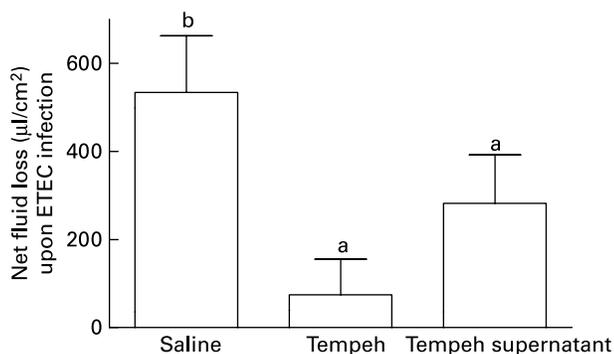


Fig. 1. Fluid loss upon enterotoxigenic *Escherichia coli* (ETEC) infection after perfusion with saline, pre-digested tempeh and its supernatant. Values are means with their standard errors depicted by vertical bars. ^{a,b}Mean values with unlike superscript letters were significantly different ($P < 0.05$).

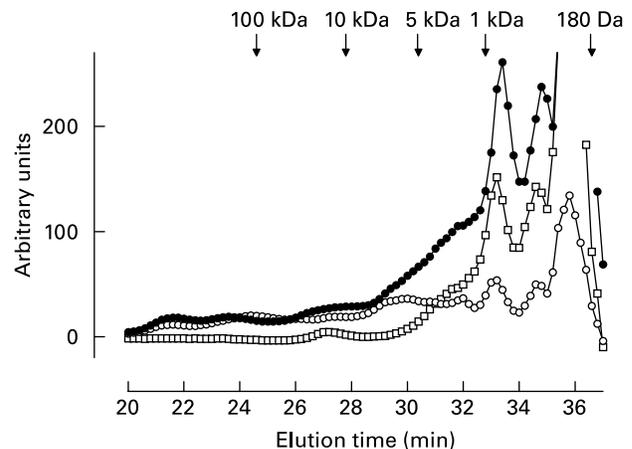


Fig. 2. High-performance size-exclusion chromatography elution patterns of supernatant of pre-digested tempeh (●) and the permeate (□) and retentate (○) fraction obtained after ultra-filtration. ↓, Molecular weights of dextran standards.

Table 1. DM, nitrogen, sodium and chloride content and osmolality of the pre-digested supernatant and the permeate and retentate fractions obtained after ultra-filtration

(Mean values with their standard errors)

	DM (g/l)	Nitrogen (g/l)			Sodium (mmol/l)		Chloride (mmol/l)		Osmolality (mOsmol/l)	
		Total	<5 kDa	>5 kDa	Mean	SEM	Mean	SEM	Mean	SEM
Supernatant	28.0	2.5	2.4	0.1	52	4	50	6	231	24
Permeate (<5 kDa)	19.4	1.6	1.6	0.0	40	8	41	7	169	34
Retentate (>5 kDa)	8.9	0.7	0.6	0.1	12	7	11	5	55	28

Discussion

Previously we demonstrated that pre-digested tempeh stimulates DM absorption and reduces ETEC-induced fluid and electrolyte losses in piglet small intestinal segments, whereas this was not the case for non-fermented soyabeans¹². The protective effect of pre-digested tempeh against ETEC-induced fluid loss appears to be determined in part by the presence of the insoluble matrix of tempeh. The presence of insoluble material could probably modify fluid absorption as has been suggested for viscosity-enhancing agents^{21,22}. Whereas pre-digested tempeh as such reduced loss in net fluid absorption by about 85% compared to saline, the supernatant of pre-digested tempeh reduced loss in net fluid absorption by about 50%.

Although low osmolality promotes net fluid absorption in both non-infected and secreting intestine^{23–25}, the difference between net fluid absorption in non-infected and ETEC-infected segments is fairly independent of osmolality²³. Therefore the lower decrease in net fluid absorption upon ETEC infection for perfusion with supernatant and retentate cannot be attributed to osmolality and we hypothesize that tempeh components interfere in the pathogenesis of the ETEC infection, resulting either in reduction of secretion or stimulation of absorption, or both.

The mechanisms whereby high molecular weight soluble tempeh compounds exert their beneficial effects in the case of the observed reduced fluid and electrolyte losses remain

to be determined. These may involve aspects of enzyme activity, interference with pathogenicity factors and stimulation of fluid absorption.

Tempeh contains a diversity of microbial enzyme activities. α -Galactosidase and protease activity were found in the supernatant of pre-digested tempeh and in its retentate but not in its permeate (JL Kiers, unpublished results). Enzymatic activity in the gut could degrade intestinal receptors for ETEC as was shown before for bromelain^{26–28}. Components in the tempeh extract could also interfere with the (binding of) enterotoxin, as was shown elsewhere for certain toxin receptor analogues²⁹ or interfere in the cascade of events leading to an increased chloride secretion and inhibited sodium chloride absorption, as was shown for polyphenolic compounds in plant extracts and boiled rice^{30–32}.

Tempeh components could also exert a pro-absorptive activity. This could be mediated through stimulated sodium-solute co-transport which is the basis for traditional oral rehydration therapy²⁴. In the present study there was a net absorption of solutes both in the tempeh supernatant as well as in the permeate and retentate. Probably this reflected the absorption of low molecular weight components such as monosaccharides and amino acids present in supernatant and permeate and in low concentrations in retentate. Since the reduction of fluid loss was found only in the retentate but not in the permeate, which contained higher levels of compounds supposed to enhance fluid absorption through sodium-solute co-transport, coupled sodium-solute absorption is unlikely to be responsible for the observed protective effect.

Vegetable polysaccharides, like starch^{33,34} and gum Arabic^{35–37}, have been shown to enhance intestinal electrolyte and/or fluid absorption in normal and secreting rat small intestine. Gum Arabic has been shown to enhance net sodium absorption without altering net fluid absorption in normal rat jejunum³⁷, whereas fluid absorption was increased in the case of diarrhoea^{35,36}. Enhanced absorption could be the result of increased accessibility of electrolytes and associated fluid to the microvillus membrane through the emulsifying properties of gum Arabic³⁶, but also other physicochemical properties could play a role³³. Evidence for the possible involvement of tempeh polysaccharides in the protective effect described in the present study could be the presence of high molecular weight polysaccharides in the tempeh supernatant and its retentate. Additionally, the high-performance size-exclusion chromatography analysis of the supernatant of pre-digested tempeh was identical to the supernatant of undigested tempeh, especially with regard to high molecular weight

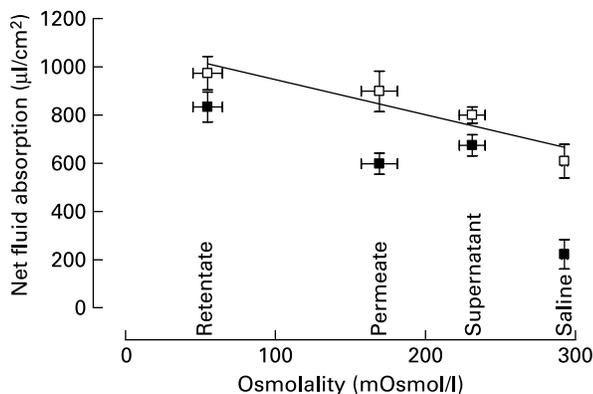
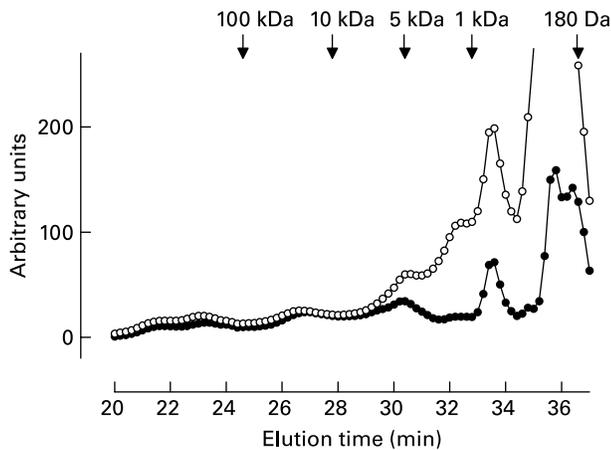


Fig. 3. Net fluid absorption in non-infected (□) and enterotoxigenic *Escherichia coli*-infected (■) segments perfused with saline and tempeh supernatant, permeate and retentate. Values are means with their standard errors depicted by bars. There was an inverse linear relationship between osmolality and net fluid absorption for non-infected segments (net fluid absorption = 1091 - 1.45 × osmolality; r^2 0.87).

Table 2. Average net absorption of sodium, chloride and solutes after perfusion with saline and tempeh supernatant, permeate and retentate (Mean values with their standard errors)

	Sodium ($\mu\text{mol}/\text{cm}^2$)				Chloride ($\mu\text{mol}/\text{cm}^2$)				Solute ($\mu\text{mol}/\text{cm}^2$)			
	Non-infected		ETEC-infected		Non-infected		ETEC-infected		Non-infected		ETEC-infected	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Saline	99 ^c	16	44 ^{c**}	8	101 ^c	16	53 ^{c**}	8	-9 ^a	4	-19 ^a	3
Supernatant	43 ^b	3	27 ^b	4	50 ^b	4	37 ^{bc}	5	85 ^c	6	69 ^d	5
Permeate (<5 kDa)	39 ^b	6	7 ^{ab*}	3	44 ^b	7	20 ^{ab}	4	70 ^c	8	40 ^{c*}	5
Retentate (>5 kDa)	15 ^a	2	2 ^a	1	16 ^a	2	7 ^a	1	16 ^b	4	6 ^b	3

ETEC, enterotoxigenic *Escherichia coli*.^{a-d} Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).Mean values were significantly different from those of the non-infected group: * $P < 0.01$; ** $P < 0.001$.**Fig. 4.** High-performance size-exclusion chromatography elution pattern of the supernatant of undigested (●) and pre-digested tempeh (○). ↓, Molecular weight of dextran standards.

compounds, and supernatant of undigested tempeh showed equal protection against ETEC-induced fluid loss as supernatant of pre-digested tempeh.

The present results warrant the protective role that tempeh could play in ETEC-associated diarrhoea and show the role of its high molecular weight soluble fraction. Further research is required to identify the component(s) in the high molecular weight soluble fraction which is responsible for the protective effect and to evaluate the specific mechanism(s) underlying the improved net fluid balance.

Table 3. Nitrogen content and osmolality of undigested and pre-digested supernatant of tempeh

	Nitrogen (g/l)			Osmolality (mOsmol/l)	
	Total	<5 kDa	>5 kDa	Mean	SEM
Undigested	0.7	0.6	0.1	67	1
Pre-digested	2.0	2.0	0.1	217	1

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