Effects of fermented soya bean on digestion, absorption and diarrhoea

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 25 september 2001
des namiddags te half twee in de Aula

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Keywords: soya bean, fermentation, tempe, *Rhizopus*, *Bacillus*, digestibility, *E. coli*, ETEC, absorption, intestinal perfusion, pig, diarrhoea

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Stellingen

1. Gefermenteerde soja is licht verteerbaar en beschermt in geval van diarree. Daarom is gefermenteerde soja bij uitstek geschikt als voeding voor jonge, ondervoede individuen met diarree

   Dit proefschrift

2. Infectie met enterotoxigene *Escherichia coli* (ETEC) leidt tot een aanzienlijke reductie in netto vloeistofopname onafhankelijk van de osmolaliteit van het darmlumen

   Dit proefschrift

3. De beste sojaboon is een 'uitgekookte' sojaboon

   Dit proefschrift

4. Voor pathogene micro-organismen zijn wij niets anders dan zachte, dunwandige flessen met cultuurmedium

   Levin, BR & Antia, R (2001) Why we don't get sick: The within-host population dynamics of bacterial infections. *Science* **292**: 1112-1114

5. De rol van stikstofmonoxide in de water- en elektrolytenhuishouding in de darm is onbegrijpelijk

   Mourad, FH, Turvill, JL & Farthing, MJG (1999) Role of nitric oxide in intestinal water and electrolyte transport. *Gut* **44**: 143-147

6. Let Food be Thy Medicine

   Hippocrates

7. Het venijn van een haai zit in zijn vin

   Trouw, 4 juli 2001

Stellingen behorende bij het proefschrift:

'Effects of fermented soya bean on digestion, absorption and diarrhoea'

Jeroen Kiers

Wageningen, 25 september 2001

Contents

Abstract

For many centuries Asian people have consumed soya beans in various forms of traditional fermented soya bean foods. Major desirable aspects of fermented soya bean foods are their attractive flavour and texture, certain nutritional properties, and possible health promoting effects. This study describes effects of fermented soya beans on gastrointestinal physiology and addresses digestion, absorption and diarrhoea.

Using an *in vitro* digestion model it appeared that fermentation increased solubility and absorbability to a large extent as a result of protein and carbohydrate degradation. The level of water-soluble dry matter increased during fermentation with *Rhizopus* sp. (tempe) from an initial 7 up to 27%, and during fermentation with *Bacillus* sp. from an initial 22 up to 65%. *In vitro* digestibility was only slightly higher for the fermented soya beans. Soya beans are more or less pre-digested by the action of the micro-organisms and can therefore serve as a source of easily available nutrients.

Tempe extracts did not inhibit the growth of *Escherichia coli.* Enterotoxigenic *E. coli* (ETEC) induced haemagglutination of red blood cells was strongly inhibited by tempe extracts, and *in vitro* adhesion of ETEC to brush border membranes isolated from the small intestine of piglets was inhibited up to 95% by several tempe extracts.

Perfusion of small intestinal segments of anaesthetised piglets showed an inverse relationship between osmolality and net fluid absorption (linear correlation). ETEC infection of small intestinal segments prior to perfusion resulted in an osmolality independent reduction of net fluid absorption of approximately 400 $\mu l/cm^2$. Both cooked soya bean and tempe were able to minimise this reduction in net fluid absorption induced by ETEC. However, sodium losses as a result of ETEC infection were lower and dry matter and total solute absorption were higher for tempe when compared to cooked soya bean. A fraction containing high-molecular-weight components (> 5kDa) was shown to be mainly responsible for the observed protective effect of tempe. Several possible mechanisms of action are outlined and discussed.

Soya beans fermented with *Rhizopus microsporus* showed better protection against ETEC-induced diarrhoea compared to cooked and especially toasted (commercial) soya beans in weaned piglets *in vivo*. Furthermore, fermentation of cooked soya beans, especially with *Bacillus subtilis*, resulted in increased feed efficiency probably as a result of increased digestibility. These characteristics imply the potential of using fermented soya beans in individuals suffering from diarrhoea and malnutrition.

Voorwoord

En daar is ie dan! Na het eerste jaar wist ik niet of ik het af zou kunnen maken, na het tweede jaar wist ik niet of ik het nog wel af wilde maken, na het derde jaar wist ik niet of ik het ooit af zou krijgen, na het vierde jaar was het merendeel afgerond en dan nu na precies vijf jaar is het echt af! De resultaten uit deze periode van promotieonderzoek staan in dit boekje: het proefschrift. Hierin is mijn onderzoek naar het effect van gefermenteerde soja op vertering, absorptie en diarree beschreven. Aspecten uit de levensmiddelentechnologie, voeding, microbiologie en (dier)fysiologie bepaalden de dagelijkse werkzaamheden, variërend van het opkweken van bacteriën en schimmels tot het opereren van biggen en het koken van honderden kilos sojabonen. Dit had ik natuurlijk nooit allemaal in mijn eentje kunnen en willen doen: promoveren is een teamsport. Ik wil dan ook de volgende teamleden bedanken:

Rob, Marius en Klaske bedankt voor het initiëren van het project en het halen van een nieuwe speler naar de vakgroep Levensmiddelenmicrobiologie. Mijn promotor Prof. Rombouts, aanvoerder van het team, bedankt voor je betrokkenheid en ondersteuning in de afgelopen vijf jaar. Mijn copromotoren, Rob Nout en Marius Nabuurs, die vanuit de achterhoede het spel controleerden en af en toe een scherpe voorzet gaven. Rob, jij bent een rots in de branding geweest, bedankt voor je nuchtere kijk op de verschillende aspecten van en je prettige begeleiding tijdens het onderzoek. Marius, bedankt voor je kritische en inspirerende woorden en je peptalks als het eens tegen leek te zitten. Jouw manier van enthousiasmeren is onnavolgbaar! Jan van der Meulen, als waardevolle aankoop halverwege het seizoen ben je uitgegroeid tot een meer dan volwaardig en belangrijk onderdeel van het team. Bedankt voor alle uren van analyses vóór, tijdens en na de vele onderdelen van het spel. Zonder een sponsor kan een team niet presteren: Numico bedankt voor de financiële ondersteuning.

Om als team te slagen zijn vele spelers nodig. Belangrijke spelers op het centrale middenveld waren Arie, Esther, Jan, Ank en Gertjan, die mij altijd, tot in de kleine uurtjes, hebben geholpen bij het uitvoeren van de SISP-testen. Onwijs bedankt hiervoor! Arie, bedankt voor het verzorgen van de cooling-down na een zware wedstrijd, als we samen de prestaties van de Nederlandse voetbalcompetitie onder het genot van een biertje (en een warme hap uit de 'stoof') bespraken. Tot slot alle overige medewerkers van ID-Lelystad, die als invaller of verzorger een waardevolle toevoeging aan het team (ook tijdens de uitwedstrijden!) vormden, Theo, Ad, Ad, Dirk, Dik, Petra en Fred en de rest van IPE, DB en Bacteriologie. Bedankt!

Een team is niets zonder een goed functionerende rechterflank. Het team kon daarbij putten uit een groot aantal mensen in het bijzonder mijn kamergenoten Ralf en Graça, die de sfeer in de ploeg hielden, Birgit die ondersteunde bij de analyses en onze Chinese international Beizhong: Thanks for the wonderful time! Graça thanks for being my paranimf. Een aantal (internationale) junioren kreeg een kans zijn/haar spel te laten zien en wist daar ook zeer goed gebruik van te maken: Carles Prior Pociello, Jisvi Schreuder, Lena Lycken, Gerard Schrijer, Leontine van der Burgt, Ivon Milder, Pascale Geerts, Paulien Hollering, Sharon Sriram, Annick van Laeken, Sergio Lopez Moscat, Mirjana Eimermann en Jens Schlößer: Thanks for your valuable participation and good luck in the future! De rechterflank werd ondersteund door het secretariaat, de materiaalmannen van de werkplaats, iedereen van Food Microbiology en Food Chemistry, en de overige spelers uit het Biotechnion. Allemaal ontzettend bedankt hiervoor!

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Een team valt of staat bij de steun van zijn supporters. Deze supporters hebben gedurende de afgelopen vijf jaar gezorgd voor de nodige afleiding, ontspanning en ondersteuning. Ook deze mensen mogen niet vergeten worden. Allereerst de supporters die zorgden voor de sportieve ontspanning. Collega-voetballers, zowel in de zaal (Micro/Proceskunde) als op het veld (Micro-Chemie): ik heb altijd met erg veel plezier een balletje met jullie getrapt. Ook aan andere sportieve evenementen zoals het roeien heb ik met veel plezier deelgenomen. Allemaal hartstikke bedankt voor de (ont)spannende momenten. Mijn supportersgroep had ook een meer sociale kant die bestond uit borrels, etentjes, feestjes en uitstapjes met collega's (aan het einde van het seizoen aangevuld met nieuwe collega's van Unilever), vrienden en familie. Dit vormde telkens weer een welkome afwisseling op het proefschriftproces, bedankt hiervoor. Verder wil ik Eric bedanken voor het luisterend oor tijdens de biertjes in de kroeg. Ik vind het super dat je mijn paranimf wil zijn. Carolien, bedankt voor het ontwerpen van 'de voorkant', het is precies geworden wat ik wilde!

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Chapter 1

General introduction

FERMENTED FOODS

Fermented foods are those foods which have been subjected to the action of micro-organisms or enzymes that cause desirable biochemical changes and significant modification of the food (Campbell-Platt, 1994). The origins of fermented foods go back many thousands of years, with early evidence of the alcoholic fermentations of barley to beer and grapes to wine. Fermented foods may have started as 'natural' processes in which nutrient availability and environmental conditions selected particular micro-organisms which modified and preserved the food (Campbell-Platt, 1994). Fermented foods and beverages (Table 1.1) globally provide about 20-40% of our food supply (Campbell-Platt, 1994). Food fermentation represents one of the oldest known uses of biotechnology and plays at least five roles (Steinkraus, 1994; Steinkraus, 1996):

1. Enrichment of the diet through development of a diversity of flavours, aromas and textures in food substrates
2. Preservation through lactic acid, alcoholic, acetic acid, and alkaline fermentations
3. Biological enrichment of food substrates with protein, essential amino acids, essential fatty acids and vitamins
4. Detoxification and removal of anti-nutritional factors
5. Decrease in cooking times and fuel requirements

Table 1.1 Groups of fermented foods.

Group	Examples
1. Dairy	Cheese, yoghurt, kefir
2. Beverages	Beer, spirits, arak, sake, wines
	Coffee, tea
3. Cereals	Bread, pancakes, doughnuts
	Kenkey, ogi
4. Meat	Country ham, salami, pepperoni
5. Legumes	Soy sauce, miso, tempe, kinema, dawadawa
6. Miscellaneous	Vinegar
7. Fruits & vegetables	Pickles, olives, sauerkraut

Source: (Campbell-Platt, 1994)

SOYA BEAN FERMENTATION

During the past several decades, soya beans (*Glycine max*) have become an increasingly important agricultural commodity, with a steady increase in annual production. Currently, global production is estimated at 150 million metric tons, with the major production countries being the USA, Brazil, China and Argentina (Liu, 2000). In the USA most of the soya beans are crushed to extract oil, used almost entirely for human consumption, and the remaining defatted meal is mainly used in animal feed. Major groups of soya bean foods include soya oil, traditional soya bean foods, soya protein products (e.g. used in bakery, breakfast cereals and infant formulas), new-generation soya bean foods (e.g. meat alternatives like soya burgers), soya-enriched foods (e.g. soya snacks) and functional soya ingredients/dietary supplements (e.g. phytochemicals like lecithin, isoflavones, tocopherols and sterols) (Liu, 2000).

For hundreds or even thousands of years, Oriental people have consumed soya beans in various forms of traditional soya bean foods. Only during the recent 20 years they have made a significant inroad into Western cultures and diets (Golbitz, 1995). Traditional soya bean foods remain popular and can be classified into two categories: non-fermented and fermented.

Non-fermented soya bean foods include soya milk, tofu, soya sprouts and others, whereas fermented soya bean foods include among others soya sauce, miso, sufu, tempe and natto.

Fermented soya bean foods

In contrast to soya sauce, where salt is used in the production, non-salted soya bean fermentation results in products in which soya bean cotyledons are recognisable and consumed as a whole. These foods can be used as meat alternatives and/or flavour enhancers.

Non-salted fermented soya bean foods find their origin in Asia. Figure 1.1 illustrates the connection between non-salted fermented soya bean foods found in Nepal & India, China, Japan, and Indonesia, and this region has been called the natto triangle (Astuti et al., 2000). In Java fungi (for instance *Rhizopus* spp.) are used to ferment soya beans, whereas in Japan, China and Nepal bacteria (*Bacillus* spp.) are applied.

An important function of the micro-organism in the fermentation process is the synthesis of enzymes, which hydrolyse soya bean constituents and contribute to the development of a desirable texture, flavour, and aroma of the product (Hachmeister and Fung, 1993). Enzymatic hydrolysis also may decrease or eliminate anti-nutritional constituents. Consequently, the nutritional quality of the fermented product may be improved.

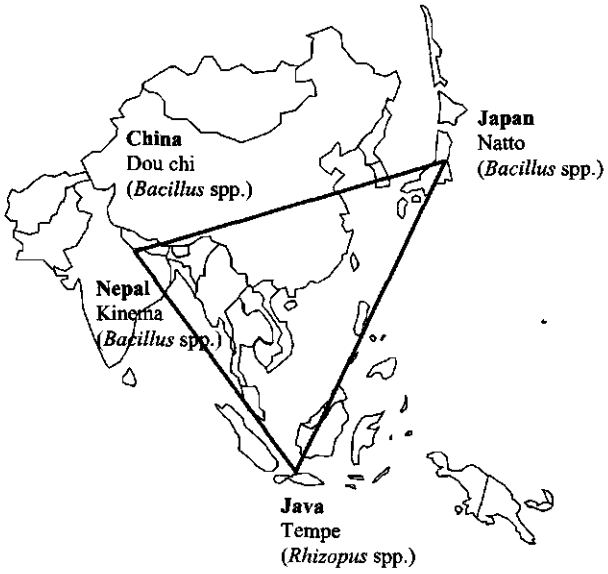

Figure 1.1 The natto triangle. Adapted from Astuti et al. (2000).

Tempe

Tempe is a traditional Indonesian fermented food in which fungi, particularly *Rhizopus* spp., play an essential role. Whereas the spelling 'tempeh' is also used, the authentic Indonesian spelling 'tempe' is used in this thesis. Fresh tempe is a compact and sliceable mass of cooked soya beans covered, penetrated and held together by dense non-sporulated mycelium of *Rhizopus* spp. (Figure 1.2). The major desirable aspects of tempe are its attractive flavour, texture and certain nutritional properties.

In Indonesia, tempe is consumed as a protein-rich meat substitute by all economic groups. The Netherlands have a sizeable population of former Indonesians who continue to produce and consume tempe as they do in Indonesia. In the United States vegetarians produce and consume tempe as a major protein source to replace meat (Steinkraus, 1996).

A range of tempe-making processes has been described for different localities and countries (Shurtleff and Aoyagi, 1985; Steinkraus, 1996). The essential stages in the preparation of tempe include: cleaning the beans, hydration/acid fermentation, dehulling, partly cooking, draining, cooling, surface drying, inoculation with the starter, incubation in fermentation containers (fermentation), harvesting and processing.

Figure 1.2 Tempe cake.

It is generally accepted that fungal growth (*Rhizopus*) is essential for tempe formation, but also that levels of 10^8-10^9 colony-forming-units (cfu)/g bacteria are common in the final product (Mulyowidarso et al., 1990). The contribution of this 'accompanying' flora of bacteria as well as yeasts to the properties of tempe is only partly understood. Most probably they play a role in flavour development and influence the chemical composition through substrate modifications and synthesis of vitamins (Keuth and Bisping, 1993; Nout and Rombouts, 1990). During the fungal fermentation stage, the mycelium of *Rhizopus* spp. penetrates several layers of cells into the soya bean cotyledon. Infiltration occurs to a depth of 2 mm in 40 hours for soya bean tempe (Varzakas, 1998). Lipases, proteases, phytases and a variety of carbohydrases are produced (Sarrette et al., 1992) and because of the enzymatic degradation of macromolecules into substances of lower molecular weight, the cell walls and intracellular material is partly solubilised (Kovac and Raspor, 1997; Nout and Rombouts, 1990) and nutritional value and digestibility might be improved.

During World War II, prisoners of war suffering from dysentery could not tolerate soya beans but were able to subsist on tempe providing early indications of increased digestibility (Anonymous, 1969; Steinkraus, 1996).

'In the latter part of 1943, the Japanese either through shortage of rice or kindness of heart, started to replace part of the rice ration with soya beans. Now soya beans is one of the best foods in the world from a nutritive point of view, and there was great rejoicing in the camp, until the hygiene officer discovered that most of the beans were being passed through the body just as they entered it, being too hard to be digested. Some Dutchmen in the camp then came to the rescue and showed us how to make tempe from the beans. This tempe is a Dutch or rather Javanese method of treating the beans and making them easily digestible. There is a fungus named Rhizopus which is found in Hibiscus flowers which abound in Singapore, and this fungus when grown on the beans softens them and makes them digestible. Before the fungus can grow on them the husks have to be removed from the beans. This is done by soaking the beans in water and then passing them between two loose rollers. In the tempe factories in Java, I am told, the natives substitute their large flat feet for these rollers, and this may be why tempe is not eaten a great deal by the Europeans there. After the husks are removed the fungus seed is mixed through the beans, which are then spread about 1" thick on trays. After about 36 hours the beans are covered with a grey furry fungus like that which grows on a piece of old cheese, and have become a solid mass now ready for cooking. It is excellent fried, baked or boiled, in fact almost anything can be done with it. Medical officers were convinced that making tempe from beans was the best way to get the most value from them. Personally, when fried, I thought tempe most tasty – something between mushrooms and pork.'
From: The Prisoner Of War Changi Vitamin Centre report by A./Sgt. C. Morton.

Kinema

Japanese natto, Thai thua-nao, Indian kinema, and certain African fermented foods such as dawadawa or soumbala represent a category of fermentations in which an alkaline pH in combination with ammonia control the fermentation (Steinkraus, 1996). The rise in pH and liberation of ammonia are related to extensive hydrolysis of protein to peptides and amino acids, and subsequent degradation of amino acids. Alkaline fermentations are dominated by bacilli, principally *Bacillus subtilis* (Antai and Ibrahim, 1986; Sarkar et al., 1994). These alkaline fermentations based upon bacilli are closely related product-wise to Oriental fungal fermentations that yield amino acid/peptide mixtures with meat-like flavours (Steinkraus, 1996). *Bacillus* fermentation of legumes reportedly resulted in improved digestibility as well (Odunfa, 1986; Sarkar and Tamang, 1995).

Kinema is a fermented soya bean food produced and consumed as a meat-flavoured, protein-rich meat substitute primarily in Nepal, Sikkim and the Darjeeling district of India (Sarkar et al., 1993). Whole soya beans are washed, soaked and cooked until soft. They may be crushed lightly to loosen the hulls. When cool, they are wrapped in large leaves in 200 to 250g portions. The inoculum usually comes from the environment. Incubation is at a warm place (35 to 45°C) for 48 to 72h. After 12h, the surfaces of the beans get covered with a rough, white, viscous microbial growth. The pH rises until the aroma becomes ammonia-like. Kinema is salted, deep-fat fried, and consumed and serves as a major source of protein in the Nepalese diet (Sarkar et al., 1997b).

General benefits of fermented soya bean

Primary benefits of soya bean fermentation are improvement of organoleptic quality and nutritional value rather than preservation. Raw soya beans are bitter in taste. Consecutive stages of the tempe fermentation process such as soaking, leaching and enzymatic modification result in the removal of the beany flavours (Nout and Rombouts, 1990). Development of flavours and aromas through fermentation is a major characteristic of

fermented soya bean foods (Steinkraus, 1996). Texture dramatically changes during fungal fermentation leading to a cake-like product with a meat-like texture. In case of *Bacillus* fermented soya bean the quality of the product is determined by its flavour and the viscous material produced (Sarkar and Tamang, 1994).

Raw soya beans contain significant levels of so-called anti-nutritional factors (ANFs), however some of these bioactive molecules are considered to have potential health effects as well (Anderson and Garner, 2000). Many of the ANFs are leached out or destroyed during soaking and cooking of the soya beans (Nout and Rombouts, 1990), but also during fermentation several ANFs like phytates are removed (Tawali et al., 1998). Micro-organisms have been shown to break down flatulence-causing non-digestible oligosaccharides, such as stachyose and verbascose (Rehms and Barz, 1995; Sarkar et al., 1997a).

Soya bean fermentation has been shown to improve the bioavailability of dietary zinc and iron (Hirabayashi et al., 1998; Kasaoka et al., 1997; Macfarlane et al., 1990; Moeljopawiro, 1986) and can have significant effects on the formation of vitamins (Bisping et al., 1993; Denter et al., 1998; Keuth and Bisping, 1993; Sarkar et al., 1998).

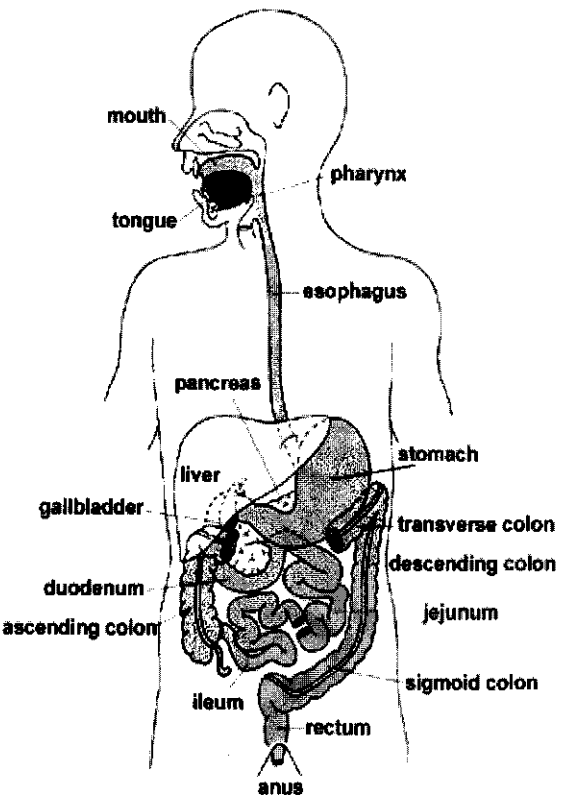

Figure 1.3 The gastrointestinal tract. Kindly provided by the Methodist Health Care System.

GASTROINTESTINAL TRACT PHYSIOLOGY

The gastrointestinal tract is the most important organ in view of the nutrition of the body. It has essential functions in digestion and absorption, but also has an important barrier function (e.g. against pathogenic micro-organisms). The gastrointestinal tract (Figure 1.3) includes the mouth, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (colon), rectum and anus.

Digestion and absorption

The principal functions of the gastrointestinal tract include digestion (degradation) and absorption (uptake) of food components and water. Digestion mainly takes place in the upper part of the gastrointestinal tract (mouth, stomach and small intestine) whereas the major sites of absorption are the (lower) small intestine and the large intestine. Digestion consists of the enzymatic hydrolysis of large dietary molecules mainly into their monomeric building blocks. A large number of digestive enzymes (e.g. proteases, carbohydrases, and lipases,

corresponding to the three major types of foodstuffs) are involved. These enzymes are excreted by the exocrine tissues (salivary glands, secretory cells in the stomach, and secretory cells of the pancreas). The final digestion and absorption of nutrients takes place in the small intestinal mucosa.

The general organisation of the small intestine can be seen in Figure 1.4. Lining the small intestine is the digestive epithelium (Figure 1.4C), which covers the finger-like villi (Figure 1.4B), which in turn cover the circular folds of the mucosa (Figure 1.4A). The villi (about 0.5-1 mm) are each surrounded by a circular depression known as the crypt of Lieberkühn (Figure 1.4C). Functions of the crypt epithelium include epithelial cell renewal and electrolyte and water secretion. The epithelium of the villi contains large numbers of absorptive cells (enterocytes) and goblet cells. The major function of the villous epithelium clearly is absorption of nutrients. The uptake of the digested materials from the lumen of the gastrointestinal tract into the enterocytes takes place across the apical membrane. The key functions of enterocytes are illustrated in Figure 1.5. First, enzymes (E) on the apical cell membrane, characterised by an approximately 0.5 to 1.5 μm wide striated border (brush

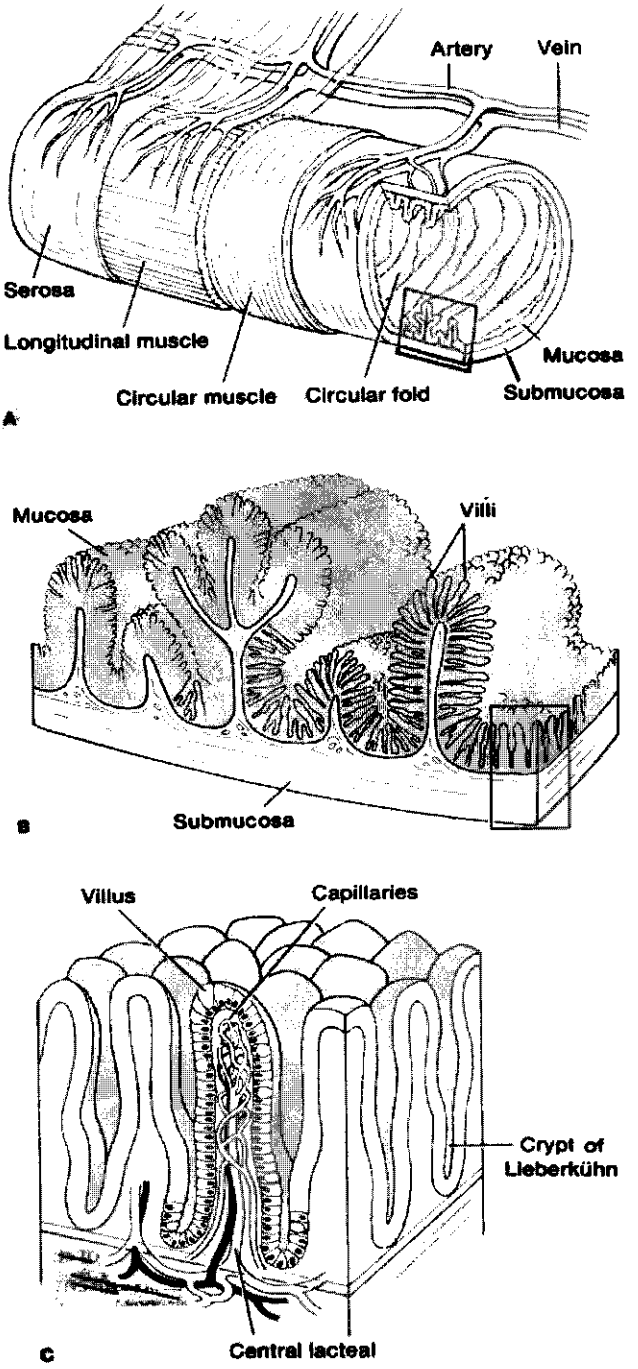

Figure 1.4 The lining of the small intestine. Source: 'The lining of the Small Intestine' by F.Moog. Copyright © 1981 by Scientific American, Inc.

border), hydrolyse dietary substrates (S) further into absorbable monosaccharides, peptides, and amino acids (Si). Secondly, specific transport processes on the brush border membrane transport sodium (Na) down an electrochemical gradient coupled to the hydrolysed substrate (Si) or chloride (Cl) into the enterocyte. The sodium pumps at the basal and lateral membranes of the enterocyte maintain a low intracellular sodium concentration and a negative intracellular electrical potential, which provides the driving force for the coupled entry processes. Sodium pumping at the lateral membranes establishes osmotic gradients for water flow into the epithelium, which normally flows along with the ions toward the capillary down a hydrostatic pressure gradient (Argenzio, 1992).

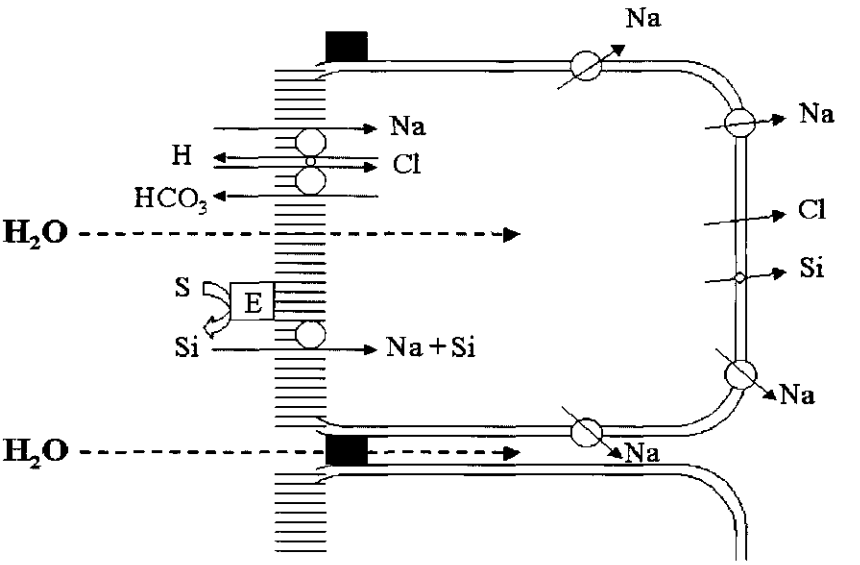

Figure 1.5 The enterocyte. Absorption of sodium coupled to chloride or solutes results in osmotic absorption of water.

Infectious diarrhoea

Malfunction of intestinal mucosa results in intestinal diseases, most of which are associated with diarrhoea. Diarrhoea is primarily a result of abnormal fluid and ion transport by the mucosa of the small or large intestine. Loss of fluid by the intestine is a result of either malabsorption and/or hypersecretion of solutes and water.

Endogenous (salivary, gastric, pancreatic, biliary and intestinal) secretions greatly exceed the daily fluid intake and may be as high as eight litres per day in adult humans. These fluids are normally recovered by the absorbing intestine. It is clear therefore that malabsorption of ions and water is sufficient to cause massive fluid losses. Such malabsorptive diseases are common and can result from virus-induced villous atrophy, or from damage to the mucosa by invading bacteria or parasites. Both maldigestion and malabsorption of substrates, ions and water occur because of the loss of brush border enzymes and transport mechanisms on the villous epithelium. Besides these malabsorptive disorders the intestine is also capable of hypersecretion, resulting in substantial loss of fluid and electrolytes. This type of fluid loss is commonly a result of colonisation of the intestinal mucosa by enterotoxin-producing bacteria, which can be accompanied by malabsorption without tissue damage.

In 1980, the yearly mortality rate for acute diarrhoea in children less than five years of age approached five million. Today, diarrhoeal diseases claim nearly two million lives a year among children under five (World Health Organization, 1999). Diarrhoeal diseases pose a heavy burden on developing countries - accounting for 1.5 billion bouts of illness a year in children under five. The burden is highest in deprived areas with poor sanitation, inadequate hygiene and unsafe drinking water. Diarrhoeal diseases are the second largest infectious killer among children under five years of age immediately after acute respiratory infections (Davey, 1999).

Enterotoxigenic Escherichia coli

In a 2-year etiological survey of acute diarrhoea in children aged 0-35 months the pathogens most strongly associated with diarrhoea were rotavirus (16% of cases, 2% of controls), *Shigella* spp. (11% of cases, 1% of controls) and enterotoxigenic *Escherichia coli* (ETEC) (16% of cases, 5% of controls) (Huilan et al., 1991). This predominance of rotavirus and ETEC as causative organisms in severe diarrhoea was confirmed recently (Bhan, 2000).

Besides the wide occurrence of ETEC in man, enterotoxic diarrhoea is also a major problem in veterinary practice (Nagy and Fekete, 1999; Van Beers-Schreurs et al., 1992). In pig husbandry ETEC is the main etiologic agent in postweaning diarrhoea and is the most common cause of postweaning mortality on many farms killing 1.5-2.0% of the piglets weaned (Hampson, 1994). Early weaned piglets show large similarities in gastrointestinal (patho)physiology to young children (Miller and Ullrey, 1987; Moughan et al., 1992). Because of these close similarities and the need to reduce diarrhoeal problems in pig husbandry, the benefits of using piglets in studying gastrointestinal disorders are obvious.

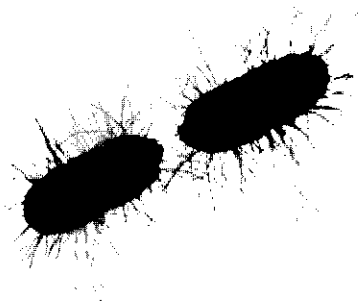

Figure 1.6 Cells of enterotoxigenic *Escherichia coli* (ETEC) with long polymeric surface proteins called fimbriae. (Copyright © by Dennis Kunkel)

The ability of ETEC to adhere to the intestinal epithelial surface of the host is an important virulence factor, and adhesion is mediated by surface appendages called colonisation factors or fimbriae (Figure 1.6) of which several serotypes have been described (Gaastra and Svennerholm, 1996; Van den Broeck et al., 2000). Once they have established in the small intestine (Jin and Zhao, 2000), ETEC produce enterotoxins that will bind to specific receptors on the epithelial cell surface. Two types of toxins are produced. The mechanisms of action and structure of heat-labile toxin (LT) are well known and are very similar to the toxin of *Vibrio cholerae*. LT binds to the GM1-gangliosides in the mucosal cell membrane and activates the epithelial adenylate cyclase system. The production of cyclic adenosine

monophosphate (cAMP) increases, resulting in increased fluid and electrolyte secretion and decreased absorption (Nagy and Fekete, 1999). Besides LT, two kinds of heat-stable toxins (ST) can be produced, STa (ST1) or STb (ST2). STa activates the guanyl cyclase system, resulting in an increased production of cyclic guanoside monophosphate (cGMP), eventually leading to the secretion of water and electrolytes (Van Beers-Schreurs et al., 1992). The mechanism of action and the molecular characteristics of STb are much less known (Nagy and Fekete, 1999). The toxins especially stimulate the crypt cells and sometimes to such a large extent that secretion totally outweighs the re-absorption, causing losses as much as 5 to 10 litres of water and salts each day in humans (Guyton, 1991). The fluids passing from the small intestine into the colon will, especially in neonates, exceed its absorption capacity resulting in diarrhoea.

Treatment

The majority of intestinal infections are self-limiting and thus the widespread, indiscriminate use of antibiotics has not to be encouraged. However, in specific circumstances antibiotics can significantly reduce the severity and duration of ETEC associated diarrhoea (Farthing, 1994). Antibiotic growth promoters have been used for more than four decades in order to increase effectiveness of livestock production (Thomke and Elwinger, 1998). Although the mechanisms of these growth-promoting antibiotics are still not fully understood, effects mediated through micro-organisms in the intestinal tract seem obvious. The continued use of the sub-therapeutic levels of antibiotics in animal feeds may contribute to the problems with antibiotic resistance in humans (Wierup, 2000) and recently this had led to restrictions in the use of antibiotics as growth promoters (Commissie Antimicrobiële groeibevorderaars, Gezondheidsraad, 1998). This strongly emphasises the need for non-pharmacological disease preventive methods as alternatives for antibiotics in veterinary practice.

Glucose-electrolyte oral rehydration solutions (ORSs) are of major importance in the treatment of acute dehydrating diarrhoeal diseases and treatment options for children with acute gastro-enteritis mainly rely on these ORSs (Farthing, 1994). In enterotoxin-mediated secretory diarrhoea, the substrate-linked sodium absorptive mechanisms (Figure 1.5) are left intact. This principle provides the rationale for the use of ORSs to rehydrate the individual. However, several problems are associated with the use of ORSs. First, they do not stop the diarrhoea (volume, frequency and duration of diarrhoea are not reduced), they simply drive a parallel process in the opposite direction. This fact may (partly) explain the widespread use of ineffective and often harmful anti-diarrhoea drugs and antibiotics (Mahalanabis, 1996). And secondly, most of the commercial isotonic solutions do not provide sufficient energy even to fulfil the maintenance energy requirement.

(Anecdotal) reports indicate that home-made starchy extracts of cereals, vegetables and other gruels are successfully used in developing countries for the treatment of diarrhoea (Molla et al., 1989). Rice-based solutions (Dutta et al., 1998; Gore et al., 1992; Iyngkaran and Yadav, 1998), carob bean juice (Aksit et al., 1998) and other food-based solutions (Molla et al., 1989) have been used to improve the effectiveness and/or acceptability of ORSs. The search for economically accessible additives to ORSs, which might provide more rapid rehydration than currently available, is ongoing. Such additives may also have nutritional value and thereby help shorten disease duration.

OPPORTUNITIES FOR FERMENTED SOYA BEANS IN IMPROVING NUTRITIONAL STATUS AND TREATMENT/PREVENTION OF INFECTIOUS DIARRHOEA

Protein-energy malnutrition is highly prevalent in the developing countries due to the decline in breast-feeding and early introduction of over-diluted commercial milk products (often contaminated during reconstitution), use of weaning foods which are low in energy and nutrients, and a high prevalence of diarrhoea and infections (Abiodun, 1991). Strikingly similar events take place in the piglet at weaning. Associated with weaning are marked changes to the histology and biochemistry of the small intestine (e.g. villous atrophy and crypt hyperplasia), which cause decreased digestive and absorptive capacity and contributes to postweaning diarrhoea (Pluske et al., 1997). Major factors implicated in the etiology of these changes include the role of enteropathogens, abrupt weaning, transient hypersensitivity to dietary antigens, withdrawal of milk-borne growth-promoting factors and low voluntary feed intake (Nabuurs, 1998; Pluske et al., 1997).

An ideal food for the prevention and management of malnutrition and diarrhoea should be of high nutritive value, easily digestible, acceptable, well tolerated and preferably should have additional anti-diarrhoeal properties.

Improvement of nutrient availability and digestibility

Early papers point out the increased digestibility of tempe (Anonymous, 1969; Van Veen and Steinkraus, 1970). *In vivo* trials using rats and neonatal pigs mostly showed either no or only slight improvements in growth, daily weight gain and protein efficiency when fungal fermented soya beans were fed compared to unfermented controls (Murata et al., 1971; Nout and Rombouts, 1990; Smith et al., 1964; Steinkraus, 1996; Zamora and Veum, 1979; Zamora and Veum, 1988). Feeding fermented soya bean could have more distinct effects in individuals suffering form a decreased gastrointestinal digestive and absorptive capacity.

Infectious diarrhoea treatment/prevention

Rabbits infected with enteropathogenic *E. coli* were fed tempe for four weeks and showed reduced diarrhoea compared to rabbits fed diets without tempe. In the tempe group no *E. coli* was found in the small intestines and histopathological examinations indicated no enteritis (Karmini et al., 1997). The effects of tempe-formulated food on the growth rate of children aged 6-24 months who were suffering of diarrhoea, was investigated and revealed beneficial effects on the duration of diarrhoea, body weight gain, and nutritional status (Karyadi and Lukito, 1996). Since *Lactobacillus* spp. (Majamaa et al., 1995; Pant et al., 1996; Shornikova et al., 1997) and *Bacillus* spp. (Kyriakis et al., 1999; Zani et al., 1998) have been used in management of acute diarrhoea, fermented soya bean foods containing high levels of lactic acid bacteria (tempe) or bacilli could be beneficial in case of diarrhoea treatment/prevention by exerting probiotic effects. It should be noted that traditionally these foods are never eaten in the raw state, and that cooking or frying prior to consumption kills the bacteria present.

The potential impact of diarrhoeal episodes on nutritional status (through the negative impacts of stool losses, vomiting, anorexia, withholding of food, and the catabolic effect of infection) seems obvious, and the synergistic interactions of diarrhoea and malnutrition are well-recognised (Bhan, 2000; Gracey, 1996). Fermented soya bean foods being nutritious, easily digested and absorbed, culturally acceptable, palatable and possibly protective against diarrhoea might break the vicious cycle of diarrhoea and malnutrition.

AIM AND OUTLINE OF THE THESIS

This thesis deals with effects of solid-substrate fermented soya bean foods on digestion, absorption and ETEC-induced diarrhoea in (models of) the gastrointestinal tract.

Fermentation is believed to improve nutritional value, nutrient bioavailability and digestibility. The digestion and absorption of food is a spatiotemporal and dynamic process involving complex enzymatic and transport reactions, and it is practically impossible to reproduce all these biochemical and physiological events in a single model (Savoie, 1994). A more practical and realistic approach is to separately evaluate the specific contributions of oral and gastric digestion, intestinal digestion by pancreatic enzymes, brush border hydrolysis, and eventually intestinal absorption and enterocyte metabolism (Savoie, 1994). In **Chapter 2** the description and use of an *in vitro* digestion model is presented. The effect of processing and (fungal) fermentation of food substrates on solubility, absorbability and digestibility is described. **Chapter 3** deals with the effect of fermentation of soya beans with *Bacillus* spp. on *in vitro* digestibility and the breakdown of protein and carbohydrates. In both chapters possible implications of fermentation on nutrient availability in health and disease are discussed.

Reports on tempe and other fermented foods have indicated possible antimicrobial activity that could play a role in the control of gastrointestinal infections. In **Chapter 4** the *in vitro* inhibitory effect of soya bean tempe on the growth and adhesive properties of ETEC is described.

Animal models using perfusion of healthy or diseased small intestine (infected with rotavirus or exposed to cholera toxin) of rats, have been used extensively to study new and established ORSs (Farthing, 1990). Perfusion techniques allow measurement of the net flux of water and solutes in sections of the intact intestine and have been used to study the effect of ETEC on net absorption of fluid and electrolytes in the small intestine of early weaned pigs (Nabuurs et al., 1994; Nabuurs et al., 1993b). It is believed that osmolality of a food or beverage plays a critical role in fluid absorption. It was therefore important to find out and understand the effect of osmolality on net fluid absorption during perfusion of normal and infected piglet small intestine (**Chapter 5**). The effect of pre-digested processed and fungal fermented soya bean products on net absorption of fluid, dry matter, electrolytes and solutes in control and infected segments of the small intestine of early weaned piglets is described in **Chapter 6** and **Chapter 7**. Possible mechanisms of action are outlined and discussed.

Large-scale fermentations were carried out with *Rhizopus microsporus* and *Bacillus subtilis*. Cooked and fermented soya beans were incorporated into weaning piglet starter diets and the *in vivo* effects of cooked and fermented soya beans on the incidence and severity of ETEC-induced diarrhoea and feed efficiency in weaned piglets are described in **Chapter 8**.

Finally, in **Chapter 9** the findings of this thesis in relation to the effect of fermentation of soya beans on gastrointestinal physiology studied in *in vitro*, *ex vivo* and *in vivo* models are integrated and discussed.

Chapter 2

In vitro digestibility of processed and fermented soya bean, cowpea and maize

Tropical legumes, i.e. soya bean and cowpea were pre-treated and subsequently fermented using pure cultures of *Rhizopus* spp. Impact of soaking, cooking and fermentation of the legumes on their digestibility was determined using an *in vitro* digestion method. Processing of white maize included amongst others, natural lactic acid fermentation, cooking and saccharification using barley malt. An *in vitro* method was standardised to carry out comparative determinations of the dry matter digestibility of cereal and legume food samples as a function of processing conditions, without attempting to exactly mimic gastrointestinal digestion. Using this method based on upper digestive tract digestion, it was observed that digestibility of the legumes increased during cooking and fermentation. Cooking improved the total digestibility of both soya bean and cowpea from 37% to 45% and from 15% to 41% respectively. Subsequent fungal fermentation increased total digestibility only about 3% for both soya bean and cowpea. Digestibility was influenced by fungal strain and fermentation time. Cooking and subsequent saccharification using malt almost tripled total digestibility of white maize from 26% to 64%, whereas lactic acid fermentation of maize had no effects on *in vitro* dry matter digestibility. Although total digestibility of cooked legumes was only slightly improved by mould fermentation, the level of water-soluble dry matter of food samples increased during fermentation with *Rhizopus oryzae* from 7% up to 27% for soya bean and from 4% up to 24% for cowpea. These fermented products could therefore play a role as sources of easily available nutrients for individuals suffering from digestive disorders.

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INTRODUCTION

Legumes and cereals are rich in nutrients and bioactive substances, but they also contain various indigestible constituents such as non-starch polysaccharides, as well as anti-nutritional factors (ANFs) such as trypsin inhibitors and phytic acid. Various processing methods such as soaking, dehulling, germination (Khetarpaul and Chauhan, 1990; Sripriya et al., 1997), cooking (Kaankuka et al., 1996), extrusion (Dahlin and Lorenz, 1993; Marsman, 1998) and fermentation (Mital and Garg, 1990; Nout and Motarjemi, 1997; Nout and Ngoddy, 1997; Nout and Rombouts, 1990; Steinkraus, 1996) have been reported to achieve improvements of nutritional value and digestibility.

Tempe is a traditional Indonesian fungal fermented food made from dehulled, soaked, and cooked soya beans inoculated with *Rhizopus* sp.. Tempe has high levels of protein and unsaturated lipids, has a pleasant flavour and texture, and has been reported to be easily digestible (Mital and Garg, 1990; Nout and Rombouts, 1990; Steinkraus, 1996). The digestibility of tempe might be caused by enzymatic degradation of soya bean polymeric substances resulting in soluble solids, particularly soluble nitrogenous compounds, by the mould during fermentation (Mital and Garg, 1990). Occasionally other legumes or cereals are used for tempe fermentation (Djurtoft, 1982; Mital and Garg, 1990; Nout and Rombouts, 1990). A significant higher growth rate, shorter duration of diarrhoeal episodes and shorter rehabilitation period in a group of children suffering from protein energy malnutrition supplemented with a porridge containing tempe and yellow maize, compared to a similar porridge made of milk and yellow maize has been reported (Kalavi et al., 1996).

In vitro digestibility studies have been carried out previously. The methods were designed to monitor specific compounds such as water-soluble dry matter, starch, protein (Yadav and Khetarpaul, 1994; Yadav and Khetarpaul, 1995) or non-digestible materials (Lebet et al., 1998). Alternative methods were developed aimed at simulating the complete human gastrointestinal tract (Minekus, 1998).

From a food technology point of view, a laboratory method was required to enable comparative assessment of the effect of food processing parameters on solubility and enzymatic degradability of foods at intermediate processing stages as well as in the final product. We developed a modular *in vitro* digestion method that enables differentiation of various aspects of digestibility such as solubility, degradability and absorbability. This investigation reports the influence of processing and fermentation on *in vitro* dry matter digestibility of soya bean, cowpea and maize.

MATERIALS AND METHODS

Micro-organisms

Rhizopus microsporus var. *oligosporus* LU 575 (NRRL 5905) and *Rhizopus oryzae* LU 582 were grown on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping off the sporangia from a slant culture after seven days incubation at 30°C, and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone. The viable count varied between 10^5-10^6 colony-forming-units (cfu)/ml when determined on Rose-Bengal Chloramphenicol Agar (Oxoid, CM 549).

An unidentified strain of *Lactobacillus* sp. predominating in natural fermented maize was isolated and maintained on de Man, Rogosa and Sharpe (MRS) medium (Merck, 1.10661) to which 12g agar was added per litre.

Processing of soya bean and cowpea

Dehulled yellow-seeded soya beans (*Glycine max*) and non-dehulled cowpea (*Vigna unguiculata*) were soaked overnight in tapwater during three cycles of accelerated acidification (Nout et al., 1987). Subsequently, the beans and peas were washed with tapwater and cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), cooled and superficially dried at room temperature. At this stage the cooked cowpeas were mildly squeezed to release the hulls obtaining a mixture of cooked cotyledons and hulls. After inoculation with the sporangiospore suspension (1% v/w) the beans and peas (450g) were packed into hard-plastic, perforated boxes (205×90×45 mm) and incubated at 30°C for 44h.

Processing of maize

Raw white maize (*Zea mays*) was coarsely ground to a flour (Fritsch, type Pulverisette 14, Germany). Two optional processing schemes A or B were followed. Option A started with accelerated natural lactic fermentation (fermentation cycles of 24h at 30°C) of a mixture of flour and tapwater in a ratio of 40g flour to 60g tapwater. Tapwater was added to the acidified dough to adjust dry matter to 15% and the porridge was cooked for 10 min (Nout et al., 1989). Processing option B started with cooking a mixture of raw maize flour and tapwater (15% dry matter) for 10 min, followed by cooling down to 50-55°C and addition of 1% w/w of malted barley maintaining the temperature at 50-55°C during 30 min. After the saccharification the product was autoclaved 30 min at 121°C. Fermentation of the autoclaved product was carried out by inoculating the sterile porridge with a pure culture of *Lactobacillus* sp. at 10^3 cfu/g and incubation during 24h at 30°C.

Sample preparation

Samples taken from each processing stage were freeze-dried and ground (Fritsch, type Pulverisette 14, Germany) using a 1.0 mm screen. All samples were defatted by extraction with petroleum-ether (40:60) in Soxhlet extractors. The solvent was evaporated, and quantification of the total crude lipid content (CL, % of dry weight) was carried out gravimetrically.

***In vitro* digestion**

The *in vitro* digestion method consisted of two steps as outlined below and illustrated in Figure 2.1. The method was carried out with 5g of non-defatted sample as well as with the residue obtained after defatting a 5g sample.

1. Enzymatic degradation
Samples (X) were suspended in distilled water (30 ml). The samples were incubated while stirring with an α-amylase-solution (2 ml) consisting of 12500 Units/l α-amylase (Sigma A-1031), 1.5 g/l NaCl, 1.5 g/l K_2HPO_4, 0.5 g/l Na_2CO_3 (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 of stomach-medium (0.1 g/l lipase (Amano Pharmaceuticals, *Rhizopus* F-AP15), 0.125 g/l pepsin (Sigma P-6887), 3.1 g/l NaCl, 1.1 g/l KCl, 0.6 g/l Na_2CO_3, 0.11 g/l $CaCl_2$, pH 4.0) for one hour at 37°C. The pH then was adjusted to 6.0 using solid $NaHCO_3$. Finally, 10 ml of a 2% pancreatic solution (20.0 g/l pancreatin (Sigma P-1750), 5.0 g/l bile (Sigma B-3883), 5.0 g/l

NaCl, 0.68 g/l KH_2PO_4, 0.3 g/l Na_2HPO_4, 0.84 g/l $NaHCO_3$) was added and the suspensions were incubated for 30 min at 37°C. Subsequently, the suspensions were centrifuged at 3000*g* for 15 min at 4°C. The supernatant was decanted and the pellet was washed twice in 20 ml of distilled water. Supernatants obtained were pooled and aliquots were taken for dry matter content determination by drying for 24h at 80°C in triplicate, resulting in S (g). The pellet was freeze-dried to determine its dry weight P (g).

2. Dialysis

In the absence of an *in vivo* epithelial uptake situation, we adopted dialysis as a physical separation technique and we have chosen dialysis membrane of a rather standard cut-off number to distinguish solubles of relatively low and high molecular mass/size. Obviously this is not identical to the different uptake processes taking place in the small intestine.

Supernatants were transferred into dialysis tubes (Medicell Int Ltd, Visking, size 8 (25.4 mm), cut-off 12-14kDa) and dialysed against running tapwater for 15h at 4°C. Retentates were quantitatively collected and their dry matter content was determined by drying aliquots for 24h at 80°C in triplicate, resulting in R (g).

Corrections and blank

S (g) was corrected for added HCl (g) and $NaHCO_3$ (g). In the *in vitro* digestion procedure a reagent blank was included resulting in Sb (g) expressing the amount of reagent that is soluble, Pb (g) expressing the amount of reagent that is insoluble and Rb (g), expressing the amount of reagent that is retained after dialysis.

Solubility and absorbability without enzymatic treatment

To determine solubility and absorbability without enzymatic treatment, defatted samples (X) were solubilised in 30 ml distilled water, incubated while stirring at 37°C for 30 min and centrifuged as described above, resulting in Su (g) and Pu (g). Supernatants were dialysed as described above. The dry matter content of the retentate obtained was determined as described earlier and resulted in Ru (g).

Calculations

In the absence of an *in vivo* situation we arbitrarily defined the following parameters. **Solubility** was defined as the percentage of a defatted sample that is water-soluble [Su/X*100%] or [(X-Pu)/X*100%]. The percentage of a defatted sample that was able to pass the dialysis tube, without being treated with digestive enzymes was defined as **absorbability** [(Su-Ru)/X*100%] or [(X-Pu-Ru)/X*100%]. Similarly, the percentage of a defatted sample that was able to pass the dialysis tube after enzymatic degradation was defined as **digestibility** [((S-Sb-HCl-$NaHCO_3$)-(R-Rb))/X*100%] or [(X-(P-Pb)-(R-Rb))/X*100%] and in case of non-defatted samples these two formulas defined **total digestibility**. In case of defatted samples, **total digestibility** was expressed as the sum of the crude lipid (CL, %) and digestibility (%) [CL+((100-CL)/100*digestibility)] assuming a 100% digestion of CL.

As shown above all parameters could be calculated in two ways, directly or indirectly. All results shown in this chapter were obtained by direct calculation. Indirect calculations were performed to confirm the values obtained after direct calculations (mass balance), but these data are not shown.

Coefficients of variation

Crude lipid, solubility and absorbability values presented in this study are the result of single determinations. When interpreting the data one has to take into account the coefficients of variations of these analyses, respectively 1.7%, 3.5% and 9.0%. Digestibility determinations were carried out in duplicate.

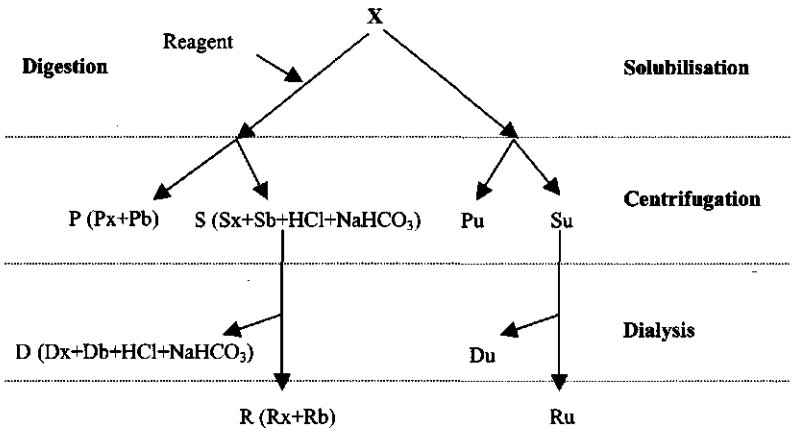

Figure 2.1 Flowsheet of the *in vitro* digestion method. All parameters are in g (dry matter): X: sample, P: pellet after digestion, Px: part of X ending up in P, Pb: part of reagent blank ending up in P, S: supernatant after digestion, Sx: part of X ending up in S, Sb: part of reagent blank ending up in S, HCl: amount of HCl added, $NaHCO_3$: amount of $NaHCO_3$ added, Pu: pellet after solubilisation, Su: supernatant after solubilisation, D: dialysate of S, Dx: part of X ending up in D, Db: part of reagent blank ending up in D, Du: dialysate of Su, R: retentate of S, Rx: part of X ending up in R, Rb: part of reagent blank ending up in R, Ru: retentate of Su.

RESULTS

pH changes during processing

Changes of pH during processing of soya bean and cowpea are shown in Table 2.1. Soaking with the accelerated acidification technique resulted in a pH of 4.2 and 4.1 for soya bean and cowpea, respectively. Cooking resulted in an increase of pH to 4.9 for both substrates. Fungal fermentation generally resulted in a pH increase as illustrated by maximum pH levels of 6.7 and 5.8 obtained in soya bean and cowpea fermented with *Rhizopus oryzae* LU 582 and *Rhizopus oligosporus* LU 575, respectively. Although not necessarily correlated, these pH changes were in accordance with comparative fungal growth activities that were measured by evolution of metabolic heat (data not shown).

Accelerated natural fermentation of maize by back-slopping resulted in a pH of 3.7 (Table 2.2). Using a starter culture of *Lactobacillus* sp. a similar pH decrease was seen after fermentation of the autoclaved material for 24 at 30°C (final $\log_{10}$ (cfu/g) =10.4).

Crude lipid content

Percentages of crude lipid content on dry weight basis are shown in Tables 2.1 and 2.2. Due to soaking and cooking of soya bean, the crude lipid content increased markedly. Fermentation of soya bean with LU 575 slightly increased the crude lipid content to 29.5% and for LU 582 it decreased to 25.9%. Fermentation of cowpea with these moulds appeared to increase the total crude lipid content, even up to 4.8% for LU 582.

Solubility

Solubility was reduced markedly in soaked and cooked soya beans and cowpeas (Table 2.1). During subsequent fermentation of soya bean and cowpea, solubility increased up to two to six times compared to the cooked samples. LU 582 resulted in higher solubility than LU 575.

Solubility of maize was hardly affected by cooking and fermentation (Table 2.2). Malting of cooked maize however resulted in a 12-fold increased solubility.

Table 2.1 Characteristics of processed soya bean and cowpea. Data represent single determinations.

	Soya bean			Cowpea		
	pH	Crude lipid (% dry matter)	Solubility (% fat-free dry matter)	pH	Crude lipid (% dry matter)	Solubility (% fat-free dry matter)
R *	6.8	20.0	64.3	6.5	0.8	30.6
S	4.2	26.5	18.7	4.1	1.6	9.8
SC	4.9	28.9	7.0	4.9	1.5	4.3
SC575, 24	5.6	28.9	18.5	5.2	1.5	13.1
SC575, 44	5.7	29.5	20.2	5.8	2.0	19.4
SC582, 24	6.4	28.2	27.3	5.0	2.2	17.0
SC582, 44	6.7	25.9	23.1	4.9	4.8	24.1

* R: raw, S: soaked, C: cooked, 575: fermented with *Rhizopus oligosporus* LU 575, 582: fermented with *Rhizopus oryzae* LU 582, 24: fermented for 24h, 44: fermented for 44h.

Table 2.2 Characteristics of processed and fermented maize.

	pH	Crude lipid (% dry matter)	Solubility (% fat-free dry matter)	Total digestibility (%)*
R **	5.6	4.0	5.6	25.5 – 25.4
S	3.7	4.0	4.6	24.2 – 24.1
SC	3.8	2.8	5.4	54.3 – 52.3
C	5.7	2.8	6.1	53.1 – 54.5
M	5.8	3.4	73.6	64.1 – 63.2
CMA	5.9	3.4	70.9	66.3 – 67.2
CMAL	3.8	3.6	68.9	67.1 – 66.5

* Duplicate values.

** See Table 2.1 and M: malted, A: autoclaved, L: fermented with *Lactobacillus* sp.

Absorbability and digestibility

In Table 2.3 absorbability and digestibility of processed and fermented soya bean, cowpea and maize are shown. For soya bean and cowpea both absorbability and digestibility decreased after soaking. However, in cooked samples digestibility was increased whereas absorbability continued to decrease. Fermentation of soya bean resulted in considerable increases of absorbability, but digestibility only increased slightly. For cowpea this was even more pronounced. Whereas cooking of maize resulted in high digestibility, absorbability did not increase until saccharification of cooked maize was conducted.

Total digestibility

In the processed soya bean samples the crude lipid contributed most to the total digestibility (Tables 2.3 and 2.4). Highest contribution to the total digestibility of cowpea samples was derived from the digestibility (absorbable material after enzymatic degradation) component.

Table 2.3 Absorbability (Abs), digestibility (Dig) and part of the digestible material that was already absorbable before enzyme treatment expressed as the ratio between Abs and Dig (A/D) for processed and fermented soya bean, cowpea and maize. Digestibility represents the average of duplicate analysis.

	Soya bean			Cowpea				Maize		
	Abs (%)	Dig (%)	A/D	Abs (%)	Dig (%)	A/D		Abs (%)	Dig (%)	A/D
R *	19.7	28.3	0.7	13.4	26.1	0.5	R	3.7	22.4	0.2
S	13.7	13.7	1.0	8.1	14.1	0.6	S	3.7	21.0	0.2
SC	4.8	22.3	0.2	3.5	40.0	0.1	SC	3.9	52.0	0.1
SC575, 24	7.6	23.7	0.3	8.1	41.7	0.2	C	3.3	52.5	0.1
SC575, 44	9.5	26.1	0.4	13.0	40.3	0.3	CM	52.4	62.4	0.8
SC582, 24	16.4	26.2	0.6	12.8	42.5	0.3	CMA	51.6	65.6	0.8
SC582, 44	14.0	27.2	0.5	18.6	39.4	0.5	CMAL	48.0	65.6	0.7

* See Tables 2.1 and 2.2.

The decrease of total digestibility due to soaking was followed by a major increase due to cooking. During soya bean fermentation with LU 575 total digestibility increased up to 47.9% after 44h. A similar increase of total digestibility was reached more rapidly by fermentation with LU 582. Prolonged fermentation of soya bean with LU 582 resulted in a decrease of total digestibility, caused by a considerable reduction of the crude lipid fraction. Fermentation of cowpea for 24h increased total digestibility with 1.6% and 2.9% for LU 575 and LU 582, respectively. During the following 20h of fermentation the total digestibility showed a decrease of 1.1% and 1.5% for LU 575 and LU 582 respectively, caused by decreases of digestibility.

Total digestibility of processed maize is shown in Table 2.2 and 2.4. Cooking resulted in the biggest increase of total digestibility, but subsequent saccharification using malt also significantly improved total digestibility. Fermentation had only minor effect on total digestibility.

In vitro digestion of defatted vs. non-defatted samples

In Table 2.4 total digestibility of defatted and non-defatted samples is shown. The data show that the values for defatted soya bean samples are much higher than for non-defatted samples. In case of cowpea and maize, values for total digestibility of defatted and non-defatted samples are less different.

Table 2.4 Total digestibility (% dry matter) of defatted and non-defatted soya bean, cowpea and maize.*

	Soya bean		Cowpea			Maize	
	Defatted	Non-defatted	Defatted	Non-defatted		Defatted	Non-defatted
R**	40.2 – 45.0	22.1 – 23.0	27.5 – 25.8	27.6 – 25.4	R	25.5 – 25.4	23.7 – 23.6
S	37.1 – 36.0	10.9 – 12.4	15.9 – 15.0	16.8 – 17.7	S	24.2 – 24.1	20.8 – 21.6
SC	44.1 – 45.4	18.5 – 16.2	40.8 – 41.0	39.9 – 38.9	SC	54.3 – 52.3	50.5 – 50.8
SC575, 24	46.0 – 45.5	16.4 – 17.3	42.3 – 42.8	43.3 – 41.9	C	53.1 – 54.5	49.6 – 48.9
SC575, 44	47.9 – 47.8	18.7 – 18.2	41.3 – 41.6	41.8 – 40.3	CM	64.1 – 63.2	62.8 – 62.2
SC582, 24	46.9 – 47.1	19.1 – 20.6	43.6 – 44.0	45.2 – 44.0	CMA	66.3 – 67.2	65.0 – 64.3
SC582, 44	45.5 – 46.5	18.9 – 20.7	42.1 – 42.5	39.9 – 39.7	CMAL	67.1 – 66.5	64.5 – 64.6

* Duplicate values.

** See Tables 2.1 and 2.2.

DISCUSSION

Loss of water-soluble solids during soaking and cooking is reflected in a decrease of solubility. Non-lipid water-soluble dry matter is lost during soaking and cooking. In addition, loss of protein into the soaking water may be enhanced by the low pH during soaking. As a result of these dry matter losses a relative increase in crude lipid content is seen. Taking into account the coefficient of variation of the crude lipid determination (1.7%), increased values of crude lipid of soaked and cooked legumes would reflect total dry matter losses of 28-33% for soya beans and 48-55% for cowpeas during soaking and cooking. The value found for soya bean is in accordance with dry matter losses observed in our laboratory. The actual dry matter losses in cowpea are in the order of 25%, implying that at low crude lipid contents estimations of dry matter losses are less accurate.

Although the dry matter losses might seem a waste, soaking is essential to increase the moisture content and leach out several ANFs and cooking is essential to destroy potential pathogenic bacteria, to inactivate trypsin inhibitors and to release some of the nutrients essential for mould growth (Steinkraus, 1996).

Acidification during the soaking of maize flour resulted in a sufficient pH decrease to contribute significantly to food safety. Upon fermentation of maize and other cereals, nutritional benefits like degradation of ANFs, increased mineral bioavailability, improvement of protein digestibility and degradation of flatulence-causing oligosaccharides may result (Nout and Motarjemi, 1997).

Tempe fermentation of soya bean and cowpea

During tempe fermentation the fermenting micro-organisms, mainly *Rhizopus* sp., induce many compositional changes, including degradation of protein, lipid and carbohydrates, catalysed by a variety of fungal proteases, lipases and carbohydrases (De Reu, 1995). In addition a decrease of ANFs is associated with the action of the moulds and their enzymes (Reddy and Pierson, 1994).

Generally, a pH increase is observed resulting from protein degradation and liberation of ammonia (Steinkraus, 1996). There are however significant differences between *Rhizopus* spp. and between the substrates in regard of pH changes and substrate modifications (Kiers et al., 1997). This is also shown by our present data.

Changes in crude lipid content during fermentation might result from metabolic activity such as assimilation and synthesis, as well as from changes of non-lipid dry matter (De Reu, 1995; Ruiz-Teran and Owens, 1996).

Solubility

The less pronounced decrease of solubility in case of cowpea could be attributed to the presence of the cowpea seed coat acting as a barrier to diffusion. A marked increase of water-soluble compounds as was shown after fermentation using *Rhizopus* sp. has been reported earlier for tempe fermentation (Van Buren et al., 1972). The high increase of solubility due to the saccharification of cooked maize can be explained mainly by the transformation of gelatinised starch into water-soluble dextrins, oligosaccharides, maltose and glucose.

Absorbability

The increase of absorbability due to mould fermentation and saccharification of maize was very likely due to the breakdown of macromolecular substances into water-soluble low-molecular-weight molecules, being small enough to pass the dialysis membrane without the need for further breakdown by gastrointestinal enzymes.

Total digestibility

We assume that the high crude lipid fraction found in soya bean is associated with the big difference in total digestibility of defatted and non-defatted soya bean. These differences were less pronounced in the case of cowpea and maize. In order to obtain maximum levels of total digestibility we recommend to defat soya bean samples prior to *in vitro* digestion.

The big increase of total digestibility of maize and cowpea is probably due to the gelatinisation of the starch, present in large amounts in these materials, making it more readily available for the enzymatic degradation. Processing of cowpea is essential because ANFs in raw cowpea could result in pathophysiological changes of gut morphology leading to impaired absorption of nutrients and consequently a diminished growth of e.g., weaning pigs (Makinde et al., 1996).

Although tempe fermentation did not increase the (total) digestibility of soya bean and cowpea to a large extent, it was shown that the amount of absorbable matter without enzymatic degradation (absorbability) increased markedly due to mould fermentation. From the ratio between absorbability and digestibility it was shown that mould fermentation attributed up to 50-60% of the digestibility. From this it can be concluded that mould fermentation already 'pre-digested' the material to a significant extent. Similarly, after saccharification of cooked maize with malt hardly any additional absorbable material is produced after incubation with gastrointestinal enzymes. Saccharification using malt was confirmed to be a suitable technique for the preparation of highly digestible, nutrient dense cereal porridges (Mensah et al., 1995). These findings have particular relevance for their use in formulated foods for individuals suffering from a malfunctioning gastrointestinal physiology.

In vivo trials using rats and neonatal pigs showed only minor differences in growth, daily weight gain and protein efficiency when fermented soya beans and cowpeas were fed compared to unfermented controls (Murata et al., 1971; Nout and Rombouts, 1990; Smith et al., 1964; Steinkraus, 1996; Zamora and Veum, 1988). This could lead to the conclusion that fermentation of legumes using *Rhizopus* spp. would hardly improve their *in vivo* digestibility in healthy rats and piglets. However, the small increase of *in vitro* digestibility and even more the large increase in absorbability caused by fermentation observed in this study could have beneficial physiological effects in case of malfunctioning gastrointestinal digestive systems. These foods are therefore expected to offer benefits when feeding weaning pigs, or children

suffering from malnutrition and/or acute diarrhoea for whom the need for easily digestible rehabilitation foods is high. The use of tempe in rehabilitation of children suffering from protein-energy malnutrition in Indonesia has already been reported (Karyadi et al., 1990) and showed a greater nutritional impact than food mixtures containing cooked soya beans.

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Chapter 3

In vitro digestibility of *Bacillus* fermented soya bean

Bacillus fermented legume products include among others dawadawa and soumbala made from African locust beans, and natto and kinema made from soya beans. *Bacillus subtilis* is the dominant species involved in the fermentation. During *Bacillus* fermentation for 48h of autoclaved soya beans the quantity of soluble and dialysable matter increased from 22% and 6% up to 65% and 40%, respectively. Protein and carbohydrate degradation during fermentation of soya bean with several *Bacillus* spp. was investigated and appeared to be substantial during the first 18h of fermentation resulting in the release of high levels of peptides and oligosaccharides. *In vitro* digestibility was increased from 29% up to 33-43% after *Bacillus* fermentation for 48h. It was shown that *Bacillus* spp. were able to degrade soya bean macromolecules to a large extent resulting in water-soluble low-molecular-weight compounds. *In vitro* digestion of *Bacillus* fermented soya bean using gastrointestinal enzymes only slightly increased the amount of dialysable matter, which clearly demonstrated the beneficial effect of *Bacillus* fermentation on food nutrient availability.

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INTRODUCTION

Bacillus subtilis is an important starter culture for Asian and African fermented soya bean foods like Japanese natto, Thai thua-nao, Indian kinema and West African dawadawa (Steinkraus, 1996). These fermentations are characterised by extensive hydrolysis of protein to amino acids, peptides and ammonia, and a rise in pH. Although most indigenous fermented foods are the result of mixed culture fermentation, it has been shown that for kinema *Bacillus subtilis* is the dominant species (Sarkar et al., 1994).

Dawadawa is generally used as a flavouring agent rather than as a source of dietary protein or calories (Odunfa, 1986), although it is used as a meat substitute by poor families in West Africa (Steinkraus, 1996). However, kinema serves as a major source of protein in the Nepalese diet (Sarkar et al., 1997b). *Bacillus* fermentation of legumes reportedly resulted in improved digestibility (Odunfa, 1986; Sarkar and Tamang, 1995).

In this study we report the effect of fermentation of soya beans using pure cultures of *Bacillus* spp. on the solubilisation and degradation of soya bean polymeric substances that may result in increased digestibility. We used an *in vitro* digestibility model which has been described and discussed earlier (Kiers et al., 2000a).

MATERIALS AND METHODS

Micro-organisms

Five strains of *Bacillus* spp. were selected based on their predominance in kinema and soumbala samples: *Bacillus subtilis* strains B82 and B83 isolated from kinema and B91 isolated from soumbala, *Bacillus badius* strain B97 isolated from soumbala and *Bacillus licheniformis* strain B70 isolated from kinema. All isolates belonged to different genotypical clusters based on RAPD analyses (data not shown).

Bacillus spp. were maintained on nutrient agar slopes (Oxoid CM3). Before use in an experiment the strains were inoculated into brain heart infusion broth (Difco 0037-17) and incubated for 18h at 37°C. The culture was diluted in sterile distilled water with 0.85% NaCl and 0.1% peptone (PPS) to approximately 10^5 colony-forming-units (cfu)/ml.

Fermentation of soya beans

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater at 4°C to avoid fermentative acidification. Soakwater was discarded and the beans were cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), cooled and superficially dried at room temperature. Cooked soya beans (100g) were transferred into glass jars and autoclaved at 121°C for 30 min, cooled and inoculated with 5 ml of diluted culture. After mixing, the beans were fermented at 37°C for 24h and 48h using the five *Bacillus* strains separately. A second fermentation series was carried out with *Bacillus subtilis* B82 and samples were fermented for 6, 12, 18, 24, 36 and 48h as described above.

pH measurements and microbial analysis

Samples (10g) were homogenised with 90 ml of sterile water. The pH was measured in this suspension using a glass electrode. Prior to pH measurement decimal dilution series in PPS were prepared and 1 ml of the dilutions was mixed with molten (45°C) nutrient agar and

poured into plates. After solidification a covering layer of nutrient agar was applied and the plates were incubated at 37°C for 24h.

Solubility, absorbability and digestibility

Samples were freeze-dried and ground to a fine flour (Fritsch, type Pulverisette 14, Germany). All samples were defatted by extraction with petroleum-ether (40:60) in Soxhlet extractors, as this was required for use in the *in vitro* digestibility model (Kiers et al., 2000a). The solvent was evaporated, and quantification of the total crude lipid content (% of dry weight) was carried out gravimetrically.

Solubility, absorbability and digestibility were determined as described earlier (Kiers et al., 2000a). In short, residues obtained after defatting 5g of sample were suspended in 30 ml distilled water and incubated for 30 min at 37°C. After centrifugation, the dry matter content of the supernatant and pellet was determined and solubility was calculated, representing the percentage of a defatted sample that is water-soluble. Supernatants were dialysed (Medicell Int Ltd, Visking, size 8, cut-off 12-14kDa) against running tapwater for 15h at 4°C. Retentates were quantitatively collected and their dry matter content was determined. The percentage of a defatted sample that was able to pass the dialysis membrane was defined as absorbability. Similarly, after solubilisation samples were treated with α-amylase, pepsin, lipase and pancreatin. Digestibility was defined as the percentage of a defatted sample that was able to pass the dialysis membrane after enzymatic digestion.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Pharmacia PhastSystem according to the instructions of the manufacturer. To reduce the protein, in order to obtain subunits, disulphide bonds were cleaved by β-mercaptoethanol. Reduction and solubilisation of protein was obtained by head-over-tail mixing for 90 min and treatment in an ultrasonification bath at 60°C for 15 min twice (Marsman et al., 1997). Finally the sample was boiled for 15min. Runs were performed in Gradient 8-25 Phastgels. Gels were fixed and stained with Coomassie Brilliant Blue R-250.

Since the samples based on autoclaved soya bean could not be separated adequately using this method, we decided to carry out an additional fermentation experiment. In this experiment we did not autoclave the soya beans after cooking as was done for the two other fermentation series. We inoculated the cooked soya beans with *Bacillus subtilis* B83 as described before and fermented the soya beans at 37°C for 24, 48 and 72h.

Gel permeation chromatography (GPC)

Molecular weight distribution of the water-soluble dry matter was performed on a LC-10Ai HPLC (Shimadzu) equipped with a Superdex Peptide column (Pharmacia Biotech 17-5003-01) and elution at 30°C with 0.1% (v/v) trifluoroacetic acid and 30% (v/v) acetonitrile at 0.5 ml/min. Calibration was performed using a range of proteins and peptides ranging from 7000 to 200Da. The eluate was monitored using an UV detector at 200nm. On the basis of the calibration curve the molecular weight corresponding to various segments of the chromatogram was calculated. The area under the curve was determined in three segments (7000-5200Da, 5200-1100Da and 1100-200Da) and was expressed as the percentage of the total area of the three segments together. The supernatant obtained after determining the

solubility (as described before) of the samples obtained from the second fermentation series was subjected to analysis.

High performance size exclusion chromatography (HPSEC)

High performance size exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300×75 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-rad Labs) in combination with a TSK guard column (40×6 mm) and elution at 30°C with 0.2M $NaNO_3$ at 0.8 ml/min. Calibration was performed using dextrans ranging from 500kDa to 180Da. The eluate was monitored using a refractive index detector. Approximately 160 mg of defatted samples obtained from the second fermentation series was added to 10 ml 10% TCA, mixed, placed for 20h at 4°C and centrifuged to remove protein and peptides. The supernatant was subjected to analysis.

RESULTS

Growth and pH changes

The growth of the *Bacillus* spp. was rapid during the first 18-24h (Tables 3.1 and 3.2) and exceeded 10^9 cfu/g wet weight after 48h fermentation. Although two of the five strains studied, B70 and B91, showed a small decrease in pH after 24h of fermentation compared to time zero, a pH increase from 6.9 for the cooked and autoclaved beans to 7.6-8.4 after 48h of fermentation was observed.

Table 3.1 Colony count, pH, solubility, absorbability and digestibility of *Bacillus* fermented soya bean. Data represent the average of duplicate determinations.

Strain	Code	Time (h)	Colony count (log cfu/g)	pH	Solubility (% defatted dry matter)	Absorbability (% defatted dry matter)	Digestibility (% defatted dry matter)
		0	3.0*	6.9*	22.3*	6.4*	28.9**
B. licheniformis	B70	24	9.4	6.5	52.1	30.7	ND
		48	9.4	7.6	54.6	32.6	ND
B. subtilis	B82	24	9.5	7.3	59.0	31.8	ND
		48	9.7	7.7	61.1	35.2	ND
B. subtilis	B83	24	9.6	7.2	52.8	29.0	34.4
		48	9.9	8.2	60.4	37.0	42.8
B. subtilis	B91	24	9.8	6.7	51.2	25.5	32.6
		48	9.3	7.8	61.9	35.9	37.9
B. badius	B97	24	8.8	7.4	51.5	26.9	30.9
		48	9.1	8.4	64.8	39.9	34.9

* average of five time duplicate determinations; ** average of three time duplicate determinations; ND not determined.

Changes in solubility and absorbability

During fermentation the crude lipid content increased from 28.8% to 32.7-37.2% of total dry matter after fermentation for 48h (data not shown).

Water-soluble dry matter increased markedly after fermentation for 24h and exceeded 50% of the total defatted dry matter (Table 3.1). The highest increase was observed between 6 and 12h of fermentation with B82, whereas solubility increased during prolonged fermentation up to about 60% of the total dry matter for all strains studied (Tables 3.1 and 3.2). Similarly, the

quantity of dialysable dry matter (absorbability) increased during fermentation. After fermentation 30-40% of the total defatted dry matter was dialysable (Tables 3.1 and 3.2).

Table 3.2 Colony count, pH, solubility, absorbability and digestibility of *Bacillus subtilis* (B82) fermented soya bean. Data represent the average of duplicate determinations.

Time (h)	Colony count (log cfu/g)	pH	Solubility (% defatted dry matter)	Absorbability (% defatted dry matter)	Digestibility (% defatted dry matter)
0	2.9	6.9	21.1	6.5	29.5
6	6.6	7.0	20.8	6.7	27.8
12	ND	ND	43.3	20.3	33.0
18	9.2	6.8	48.0	24.7	33.2
24	9.3	7.8	52.7	25.8	29.0
36	ND	ND	56.5	29.1	30.8
48	9.7	8.3	58.5	31.4	32.6

ND not determined.

Protein degradation

SDS-PAGE analysis carried out on samples which had been cooked and subsequently autoclaved prior to fermentation resulted in a very poor separation of the soya bean protein subunits. The majority of the protein from the sample obtained at time zero could not diffuse into the gel, although samples obtained after 24h of fermentation showed hardly any protein in the top of the gel anymore (data not shown). In Figure 3.1 the SDS-PAGE profile is shown for cooked and subsequently fermented soya bean with *Bacillus subtilis* B83 for 24, 48 and 72h (lane 2-5). The major protein subunits can be clearly identified in the cooked soya bean (lane 2). Already after fermentation for 24h (lane 3) all protein subunits are degraded to a large extent, and after 72h of fermentation virtually all proteins have disappeared (lane 5).

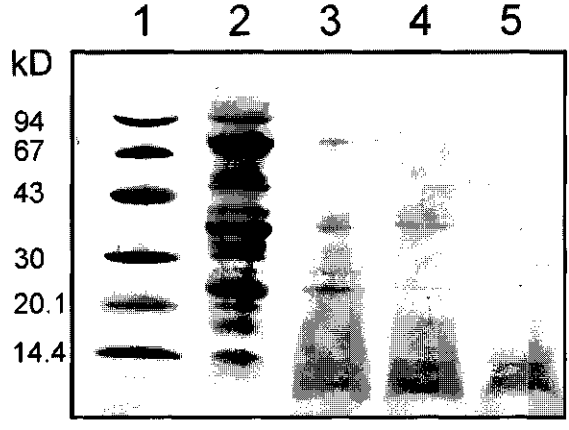

Figure 3.1 SDS-PAGE profile of fermented soya beans. In lane 1 markers of several molecular weights are shown. Cooked soya beans fermented with *Bacillus subtilis* (B83) for 0, 24, 48 and 72h are shown in lanes 2-5.

During fermentation of soya bean with *Bacillus subtilis* B82 the amount of peptides (200-1100Da) increased markedly at the expense of the protein fraction (5200-7000Da) (Figure 3.2). After 18h of fermentation only small changes were observed.

Degradation of polysaccharides

From the dextran standards used it was shown that fermentation of soya bean with *Bacillus subtilis* B82 resulted in a shift from polymeric to oligomeric matter of < 5kDa (Figure 3.3). Fermentation times over 24h resulted in an increased level of intermediate polymeric material from about 500-10kDa.

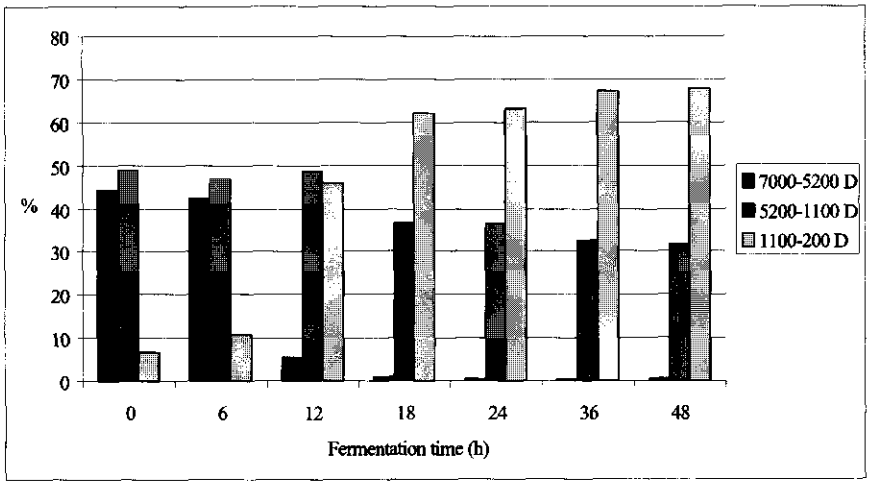

Figure 3.2 Molecular weight distribution of water-soluble protein and peptides during *Bacillus subtilis* (B82) fermentation of soya beans. Bars indicate the percentage of the total (7000-200D).

Digestibility

From Tables 3.1 and 3.2 it can be seen that digestibility increased from 29% of defatted dry matter in cooked and subsequently autoclaved soya beans to 30-40% in soya beans fermented for 48h. Compared to the values obtained for absorbability (quantity of dialysable material without enzymatic degradation) these values are only slightly higher, except for time zero (Table 3.1) and 0-18h (Table 3.2).

DISCUSSION

Changes in pH

The pH increase observed during fermentation presumably resulted from proteolysis and the release of ammonia due to utilisation of amino acids for growth. The decrease in pH upon fermentation of soya beans during the first 24h with two of the *Bacillus* spp. used could be due to the ability of these strains to initially use sugars as substrates for growth as shown before (Sarkar et al., 1993).

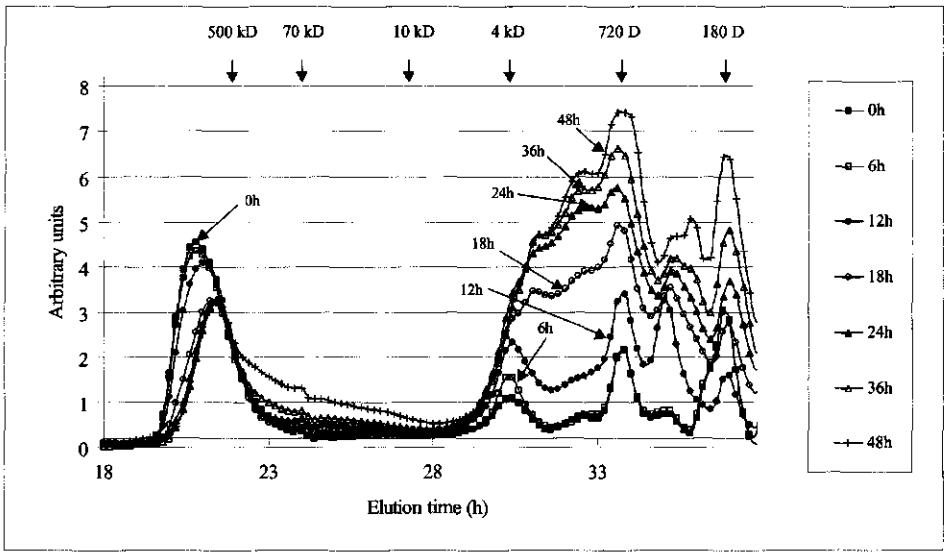

Figure 3.3 HPSEC elution pattern of water-soluble carbohydrates during *Bacillus subtilis* (B82) fermentation of soya beans. Molecular weight of several dextran standards is shown at the top of the figure.

Increase in soluble and dialysable material

We ascribe the ability of the *Bacillus* sp. to solubilise over 50% of the defatted dry matter to their high enzymatic activity. An increase in water-soluble compounds has been shown earlier for tempe fermentation where solubility increased from 7% for cooked soya bean to maximum of 27% after *Rhizopus oryzae* fermentation for 24h (Kiers et al., 2000a).

The increase in absorbability was very likely due to the breakdown of macromolecular substances into water-soluble low-molecular-weight molecules, being small enough to pass the dialysis membrane.

Degradation of protein

Soya beans contain two main storage proteins, ß-conglycinin and glycinin, which are composed of several subunits. ß-conglycinin consists of three components, the α, α' and ß subunit, whereas glycinin consists of an acidic (A) and a basic (B) polypeptide. More or less complete breakdown of all three subunits from ß-conglycinin and both polypeptides from glycinin was observed after *Bacillus subtilis* (B83) fermentation of cooked soya bean resulting in the accumulation of low-molecular-weight compounds as shown in Figure 3.1. These observations reflect the considerable proteolytic activity reported for *Bacillus* spp. (Sarkar et al., 1993; Sarkar and Tamang, 1995). Maximum proteolytic activity is reached after 12-24h and it remains fairly constant during prolonged fermentation (Allagheny et al., 1996; Sarkar et al., 1993). Processing of soya bean with *Bacillus subtilis* led to a 60-fold increase in free amino acids which accounted for approximately 26% of the total amino acid content (Sarkar et al., 1997b).

Degradation of carbohydrates

During the first 24h of fermentation, substantial quantities of polymeric matter were degraded into compounds < 5kDa, whereas during prolonged fermentation also intermediate compounds appeared. Levels of oligosaccharides like raffinose and stachyose responsible for flatus formation resulting from soya bean ingestion are reduced by soaking, cooking and *Bacillus* fermentation (Sarkar et al., 1997a). No identification of the oligosaccharides liberated during *Bacillus* fermentation was done in this study.

Digestibility

Bacillus fermentation of legumes has been associated with improvements in digestibility (Odunfa, 1986; Sarkar and Tamang, 1995). Digestibility of fermented soya beans was only slightly increased compared to the cooked and autoclaved beans, whereas absorbability was much higher for fermented soya beans. The same phenomenon was seen during tempe fermentation of cooked soya beans (Kiers et al., 2000a), although the values found for absorbability and digestibility were higher in case of *Bacillus* fermentation. In case of tempe fermentation, it was shown from the ratio between absorbability and digestibility that mould fermentation attributed up to 50-60% of the digestibility (Kiers et al., 2000a). In case of *Bacillus* fermentation this ratio was higher than 80% after fermentation for 24h increasing up to 95% after fermentation for 48h. This points to a considerable pre-digestion during *Bacillus* fermentation, leading to a product in which luminal gastrointestinal enzymes hardly can increase nutrient bioavailability.

Conclusion

Fermentation of soya bean using several *Bacillus* spp. resulted in major biochemical changes in the substrate leading to an increase in soluble and dialysable material. Investigations at protein and carbohydrate level revealed major breakdown of polymers into water-soluble low-molecular-weight peptides, oligosaccharides and monosaccharides. From the results obtained it is concluded that after 18 to 24h of fermentation considerable substrate modification had occurred leading to a product with high nutrient availability in which the need for degradation of nutrients by gastrointestinal enzymes is minimal.

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Chapter 4

In vitro inhibition of enterotoxigenic *Escherichia coli* K88 by soya bean tempe

Tempe is a traditional fungal fermented food made from soaked and cooked soya beans. It has been associated with anti-diarrhoeal characteristics. Soya beans were soaked, cooked and subsequently fermented using several *Rhizopus* spp. Water-soluble filter-sterile extracts were tested for their ability to inhibit growth of several indicator micro-organisms including *Escherichia coli* (*E. coli*). Antimicrobial activity was found against *Bacillus stearothermophilus* only. *E. coli* K88 induced haemagglutination of hamster red blood cells was strongly inhibited by tempe extracts and hardly by the cooked soya bean extract. Several tempe extracts were able to inhibit adhesion of *E. coli* K88 to piglet small intestinal brush border membranes. Tempe appeared to interfere with *E. coli* K88 adhesion *in vitro* and might therefore have a protective effect against *E. coli* K88 infection.

Submitted for publication

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INTRODUCTION

Acute infectious diarrhoea is still one of the major causes of infant and childhood mortality in developing countries, and enterotoxigenic *Escherichia coli* (ETEC) is the predominant causative micro-organism (Bern et al., 1992; Bhan, 2000). In pig husbandry ETEC is the main etiologic agent in postweaning diarrhoea (Hampson, 1994). Diarrhoea associated with ETEC involves the colonisation of the small intestine and subsequent production of enterotoxins which induce diarrhoea and dehydration. The rapid proliferation of ETEC in the small intestinal mucosa is attributable to the ability of the bacteria to attach to specific receptors present on brush borders of villous enterocytes mediated by fimbriae (e.g. K88) (Francis et al., 1999; Van den Broeck et al., 2000).

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 beans are bound together into a compact cake by dense cottony mycelium (Hachmeister and Fung, 1993; Nout and Rombouts, 1990; Steinkraus, 1996). Tempe has been reported to contain an antibacterial substance (Anonymous, 1969; Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969), has been shown to inhibit *Escherichia coli* infection in rabbits (Karmini et al., 1997; Karyadi et al., 1990) and was reported to be beneficial in terms of duration of diarrhoeal episodes and rehabilitation period when supplemented to the diet of malnourished children (Kalavi et al., 1996; Karyadi and Lukito, 1996; Soenarto et al., 1997).

These reported beneficial effects of tempe could be due to the production of antibacterial compounds against *E. coli* or interference with the adhesion of *E. coli* to the epithelial cells. The aim of this study was to determine the effect of tempe produced by different mould strains on growth and adhesive properties of ETEC.

MATERIALS AND METHODS

Moulds

Four strains of *Rhizopus microsporus* var. *oligosporus* (LU 575 (NRRL 5905), LU 2014 (NRRL 3271), LU 2021 (CBS 339.62), LU 2022 (CBS 338.62)), one strain of *Rhizopus microsporus* var. *microsporus* (LU 573) and two strains of *Rhizopus oryzae* (LU 582, LU 584 (CBS 128.08)) were grown on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping off the sporangia from a slant culture after seven days incubation at 30°C, and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone (PPS). The viable count varied between 10^5-10^6 colony-forming-units (cfu)/ml when determined on Rose-Bengal Chloramphenicol Agar (Oxoid, CM 549).

Tempe preparation

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater during three cycles of accelerated acidification (Nout et al., 1987). Subsequently, the beans were washed with tapwater and cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), cooled and superficially dried at room temperature. After inoculation with the sporangiospore suspension (1% v/w) the beans were packed into hard-plastic, perforated boxes (205×90×45 mm) and incubated at 30°C. After fermentation cooked soya beans and the products were dried at 60°C and ground using a 1.0 mm screen.

Five gram was suspended into 25 ml of phosphate-buffered saline supplemented with 1% of mannose (PBS-M) and shaken for 30 min at 400 rpm. After centrifugation (3000*g* for 15 min) the supernatant was sterilised using a 0.45 µm filter (Schleicher & Schuell FP 030/2).

Antimicrobial activity

Assays for antimicrobial activity were carried out using the agar diffusion test. *E. coli* (strains ID 1000 and ID 1017), *Micrococcus luteus* (ATCC 4911), *Bacillus subtilis* (NRRL B765/Difco 0981-36), *Bacillus subtilis* (BGA) (Merck 1.10649) and *Bacillus stearothermophilus* (Merck 1.11499) were used as indicator micro-organisms. Sterile paper disks (Difco, 1599-35) were applied to the agar surface to which the extracts were applied. After incubation clear zones around the paper disks were recorded and diameters were measured.

Haemagglutination

Hamster (*Mesocricetus auratus*) blood in EDTA (Harlan, Hont, The Netherlands) was washed five times with phosphate-buffered saline (PBS) by centrifugation at 740*g* for 10 min at 4°C. The washed erythrocytes were re-suspended in PBS to a final concentration of 2% and stored at 4°C until use.

Enterotoxigenic *E. coli* (O149:K91:K88^{ac}) strain ID 1000 and *E. coli* (O149:K91) strain ID 1084 were grown for 16-18h at 37°C in brain heart infusion broth (Difco, 0037-17). The cultures were centrifuged and the pellets were suspended in PBS-M to an absorption value of 1.00 measured at 600 nm spectrophotometrically.

The haemagglutination properties of the K88-positive (ID 1000) and the K88-negative (ID 1084) *E. coli* strain were determined as described before (Meng et al., 1998). Bacterial suspensions were serially diluted twofold with PBS-M in a V-bottom microtitre plate with 96 wells, followed by incubation with the 2% suspension of erythrocytes at room temperature for 2h. The haemagglutination titre determined was the maximum dilution of the bacteria at which erythrocytes were completely agglutinated.

The haemagglutination inhibition assay was carried out essentially as described before (Meng et al., 1998). The filter-sterile tempe extracts (25 µl) were serially diluted in PBS-M and were mixed with 25 µl of bacterial suspensions (strain ID 1000) and incubated for 30 min at room temperature with continuous gentle shaking. Afterwards, 25 µl of the 2% erythrocyte solution was mixed into each well. Plates were left standing at room temperature for 2h and inhibition of agglutination was recorded. To maximise sensitivity the test was carried out with a bacterial concentration which equalled the haemagglutination titre (Meng et al., 1998; Payne, 1994). Haemagglutination inhibitory activity (HIA) was expressed as the highest dilution of the product showing complete inhibition of haemagglutination.

K88-specific monoclonal antibodies (clone CVI F4ac-5, ID-Lelystad, Lelystad, The Netherlands) were used as a positive control. Other controls included incubation of bacteria with erythrocytes in the absence of products and incubation of products with erythrocytes in the absence of bacteria.

Brush border adhesion

Brush borders were isolated from early weaned pigs as described before (Sellwood et al., 1975). Using a Bürker-Türk counting chamber the brush border solution was diluted to 10^6 brush borders/ml. Products (25 µl) were mixed with 25 µl bacterial suspension of strain ID 1000 and incubated at room temperature with continuous gentle shaking. After 30 min 50 µl of brush borders was added and incubated at room temperature with continuous gentle shaking for 20 min. Phase contrast microscopy was used to count the number of *E. coli* cells attached to one brush border. At least 15 brush borders were examined.

RESULTS

Tempe

All mould strains tested were able to ferment the soya beans indicated by a rise in pH values after 48h of fermentation at 30°C and visual appearance of the dense cottony mycelium binding the individual soya beans into a cake-like product.

Antimicrobial effects

None of the extracts of the mould-fermented soya bean products showed any antimicrobial effect against the two *E. coli* strains tested. Growth of *Bacillus subtilis* and *Micrococcus luteus* were also not inhibited, only soya bean fermented with *Rhizopus oligosporus* LU 2014 for 48h slightly inhibited the growth of *B. subtilis* (NRRL B765/Difco 0981-36) (inhibition zone 8 mm). Several tempe extracts were able to inhibit the growth of *Bacillus stearothermophilus* (Table 4.1).

Table 4.1 Antimicrobial activity and haemagglutination inhibitory activity (HIA) of soya bean and tempe.

	Fermentation time (h)	Inhibition zone[1] (mm)	HIA[2]
Cooked soya bean		-	8
R. microsporus LU 573	48	10	128*
	96	14	256*
R. oligosporus LU 575	48	14	32*
R. oryzae LU 582	48	10	64
R. oryzae LU 584	48	-	32
	96	-	32
R. oligosporus LU 2014	48	15	*
R. oligosporus LU 2021	48	15	32*
R. oligosporus LU 2022	48	12	64
	96	13	32

[1]) Indicator micro-organism *Bacillus stearothermophilus*. According to the manufacturer only inhibition zones with a diameter larger than 10 mm should be regarded as a positive result. -) no inhibition observed

[2]) 0 = no inhibition, 1 = complete inhibition with undiluted extract, 2 = complete inhibition with two times diluted extract, 4 = complete inhibition with four times diluted extract, etc. *) interaction between product and erythrocytes in the absence of *E. coli*.

Haemagglutination inhibitory activity (HIA)

K88-positive strain ID 1000 showed strong agglutination of the hamster red blood cells, whereas K88-negative strain ID 1084 was not able to agglutinate the red blood cells. Agglutination of red blood cells by strain ID 1000 could be blocked by the K88-specific monoclonal antibodies (HIA of 512). Tempe extracts showed higher HIA compared to cooked soya bean and appeared to be different for the different mould strains (Table 4.1). Several tempe extracts showed formation of an erythrocyte mat in the absence of *E. coli* (indicated by * in Table 4.1). Except for *Rhizopus oligosporus* LU 2014, inhibition of haemagglutination occurred when these tempe extracts were diluted at least four times.

Brush border adhesion inhibition

Incubation of brush borders with *E. coli* K88-positive strain ID 1000 resulted in an adhesion of 7.5 ± 0.5 (average ± standard deviation) *E. coli* cells to one brush border (Figure 4.1). In contrast, only 0.2 ± 0.1 cells/brush border were observed in case of the K88-negative strain ID 1084. Adhesion of K88-positive strain ID 1000 was strongly inhibited by several tempe extracts (Figure 4.1). Soya beans fermented with *Rhizopus microsporus* (LU 573) and *Rhizopus oryzae* (LU 582, LU 584) showed aggregation of the *E. coli* cells.

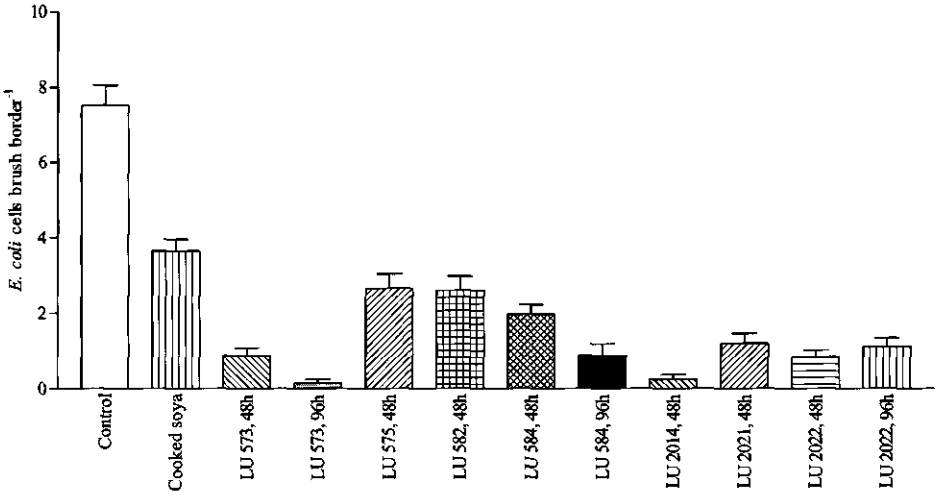

Figure 4.1 Inhibition of brush border adhesion by cooked soya bean and tempe extracts (average ± standard deviation).

DISCUSSION

None of the tempe extracts was able to inhibit the growth of *E. coli*. Some antimicrobial activity was observed against *Bacillus* sp., which is in accordance with reported antibiotic activity against Gram-positive bacteria like *Bacillus* sp. (Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969).

ETEC that express K88 fimbriae are a major cause of diarrhoea and death in neonatal and young pigs. K88 fimbriae are filamentous surface appendages that enable the adherence to

intestinal enterocytes facilitating the colonisation of the small intestine. Haemagglutination is a characteristic of many bacterial fimbriae and can be used as a model system for studying the adhesive properties of these fimbriae. Haemagglutination was reported to resemble the attachment of K88-positive bacteria to the gut wall in enteric disease (Jones and Rutter, 1974). Inhibition of haemagglutination by *E. coli* cells has been shown for galactosyl residue-containing glycoproteins including porcine stomach mucin (Meng et al., 1998) and a range of glucosides, glucosamine and pig gastric mucin (Payne, 1994). In our study tempe extracts, especially tempe produced with *Rhizopus microsporus* LU 573, showed considerable inhibition of haemagglutination.

The brush border adhesion inhibition assay concerns the actual binding of the K88-positive *E. coli* of porcine origin to isolated porcine brush border membranes. Tempe extracts were able to inhibit ETEC adhesion. Inhibition of ETEC to brush border membranes, mucus or gangliotetraosylceramide (asialo GM1) was reported before for inulin (Rossi et al., 2000) and high-molecular-weight proteinaceous factors produced by or derived from *Lactobacillus fermentum* and bifidobacteria (Blomberg et al., 1993; Fujiwara et al., 1997; Ouwehand and Conway, 1996). Tempe produced with *Rhizopus microsporus* LU 573 showed high inhibitory activity probably (partly) as a result of the ability to aggregate the ETEC cells. Aggregation of ETEC cells after incubation with the tempe extract was also shown for K88-specific monoclonal antibodies and could probably reduce ETEC attachment independent of the receptors (Francis et al., 1999; Van den Broeck et al., 2000) present in the intestinal mucosa. Besides the ability of tempe (produced with certain specific mould strains) to aggregate ETEC (antibody-like activity), tempe could also contain substances which might bind either to the fimbriae or to the receptor site resulting in inhibition of attachment.

In conclusion, certain mould-fermented soya bean products protect against the adhesion of ETEC to erythrocytes and brush border membranes *in vitro*, probably as a result of the presence of antibody-like anti-adhesins and/or adhesin analogues and/or receptor analogues. Further study is required to determine whether these compounds are liberated from the soya bean during fermentation or whether they are metabolites or cell wall compounds of the fermenting micro-organisms (*Rhizopus* sp. and/or lactic acid bacteria). Other interesting issues concern the purification and characterisation of the compound(s) as well as their stability during gastrointestinal passage. The findings presented in this study indicate a potential role for tempe in the inhibition of ETEC adhesion, which could enhance the removal of the micro-organism by normal passage of intestinal contents and therefore prevent or decrease ETEC-associated diarrhoea.

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Chapter 5

Inverse relationship of osmolality and net fluid absorption in piglet small intestine

Major problems associated with acute secretory diarrhoea are found among children in developing countries. Low osmolality is of primary importance in mediating the increased water absorption from several hypotonic oral rehydration solutions. Other factors can not be excluded since sodium and other electrolyte and solute concentrations are not uniform among the oral rehydration solutions tested. Problems with acute diarrhoea also frequently occur in pig husbandry. Pigs show large similarities in gastrointestinal physiology compared to humans. Solutions differing in composition and osmolality were used to perfuse small intestinal segments of anaesthetised piglets. A strong inverse correlation between osmolality of the perfusion fluids (150-375 mOsmol/kg) and average net fluid absorption was observed both in uninfected and enterotoxigenic *Escherichia coli* (ETEC)-infected segments. However, reducing the osmolality of the perfusion solutions below 150 mOsmol/kg hardly increased the average net fluid absorption. Replacing part of the sodium chloride by mannitol resulted in decreased net fluid absorption both in uninfected and ETEC-infected segments. It was also shown that WHO-ORS resulted in higher net fluid absorption during ETEC infection than a sodium chloride based perfusion fluid of the same osmolality. Hypotonicity is beneficial in fluid absorption in the small intestine of piglets. At low osmolality net fluid absorption is not driven by sodium absorption which suggests the existence of a physical osmotic gradient. The decrease in net fluid absorption due to ETEC infection however is independent of osmolality of the perfusion solution. The SISP model in early weaned piglets is a good model for children to test anti-diarrhoea agents and ORSs on their capability to decrease the observed difference in net fluid absorption between uninfected and ETEC-infected segments.

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INTRODUCTION

Acute infectious diarrhoea is still a major cause of childhood mortality throughout the world (Bern et al., 1992; Mathan, 1998; Rabalais, 1996), about 20% due to enterotoxic bacteria (Huilan et al., 1991). Infants and children with dehydration due to acute diarrhoea are treated effectively with oral glucose/electrolyte solutions. Evaluation of reducing osmolality in oral rehydration solutions (ORSs) revealed beneficial effects on the clinical course of acute diarrhoea in children (Bhan et al., 1995; Mahalanabis, 1996).

Animal models involving intestinal perfusion have been used quite extensively to pre-screen new ORSs (Farthing, 1990). Overall, these animal models have emphasised the potential benefit of using a hypotonic ORS (Farthing, 1990; Pillai et al., 1994a; Thillainayagam et al., 1993). Also human perfusion studies (Hunt et al., 1992; Hunt et al., 1994) in secreting intestine showed clear benefits of using ORSs with reduced osmolality. In a recent overview it was stated that the enhanced clinical efficacy of complex carbohydrate ORSs is most likely caused by their hypotonicity, although other factors can not be excluded (Thillainayagam et al., 1998).

Enterotoxic diarrhoea is also a major problem in veterinary practice, since in pig husbandry enterotoxigenic *Escherichia coli* (ETEC) is the main etiologic agent in both pre- and postweaning diarrhoea (Hampson, 1994). Early weaned piglets show large similarities in gastrointestinal (patho)physiology to young children (Miller and Ullrey, 1987; Moughan et al., 1992). By using the small intestinal segment perfusion (SISP) model (Nabuurs et al., 1993b) it is possible to perfuse small intestinal segments simultaneously within one piglet with several different solutions both in the absence and in the presence of ETEC. The aim of this study was to determine the effect of osmolality of perfusion fluids on net fluid and electrolyte absorption in uninfected and ETEC-infected small intestinal segments of piglets.

MATERIALS AND METHODS

Animals

Piglets (crossbred Yorkshire × (Large White × Landrace)) were weaned at three weeks of age. They were transported to the institute and fed a standard piglet feed. Water containing 60 mg/l colistinsulphate was applied *ad libitum*. About two weeks after weaning biopts from the duodenal mucosa were taken using a fiberscope (Olympus GIF XP10, Olympus, Hamburg, Germany) and receptor status was determined essentially as described earlier (Sellwood et al., 1975). Piglets that expressed receptors involved in binding of the ETEC strain where used in the experiments three weeks after weaning.

All experiments were conducted in accordance with the standards of the Ethic Committee of the Institute.

Bacterial strain

Enterotoxigenic *Escherichia coli* (ETEC) (O149:K91:K88^{ac}), producing heat-labile toxin (LT) and heat-stable toxin b (STb), was grown for 16-18h at 37°C in brain heart infusion broth. The culture was centrifuged and the pellet was suspended in phosphate-buffered saline (PBS) to an absorption value of 1.0 measured at 600 nm spectrophotometrically, corresponding to 1×10^9 colony-forming-units (cfu)/ml.

Perfusion fluids

Composition of the different perfusion fluids is shown in Table 5.1. In the first experiment with eight piglets sodium chloride based solutions in the range of 150-375 mOsmol/kg (C150, C225, C300 and C375) were used in two repetitive Latin Square designs. The range was enlarged to 0-600 mOsmol/kg (C0, C75, C150, C300 and C600) in the second experiment with five piglets in a Latin Square design. In the third experiment with four piglets sodium chloride perfusion fluids (C150 and C300) were compared with solutions in which half of the sodium chloride was replaced by mannitol (C150M and C300M) in a Latin Square design, while in the same experiment the sodium chloride C300 was compared to ORS formulated according to the World Health Organisation (WHO) (in g/l: NaCl, 3.50; KCl, 1.50; $Na_3C_6H_5O_7 \cdot 2H_2O$, 2.90; glucose, 20.0; osmolality of 311 mOsmol/kg).

Table 5.1 Composition of the perfusion fluids.

Code	NaCl (g/l)	Glucose (g/l)	Casamino acids (g/l)	D-Mannitol (g/l)	Measured osmolality* (mOsmol/kg)
C0	-	1.0	1.0	-	16±3
C75	2.18	1.0	1.0	-	88±1
C150	4.35	1.0	1.0	-	157±1
C225	6.53	1.0	1.0	-	227±3
C300	8.70	1.0	1.0	-	288±1
C375	10.88	1.0	1.0	-	360±1
C600	17.40	1.0	1.0	-	566±4
C150M	2.18	1.0	1.0	13.65	167±1
C300M	4.35	1.0	1.0	27.30	312±1

* values are mean ± standard deviation (n=4)
- not added

Surgical procedure

The anaesthetic and surgical procedures used were essentially as described before (Nabuurs et al., 1993b). The animal was tranquillised with 2 mg/kg bodyweight azaperon. Anaesthesia was induced with halothanum and nitrous-oxide. The abdominal cavity was opened and the first intestinal segment was prepared approximately 300 cm caudal to the stomach. A small cranial tube (inflow) was placed and a wide tube (outflow) was placed 20 cm distal from the first. Caudal and adjacent to this first segment nine other segments were prepared in the same way. In this way the ten segments were situated on average at 50% of the total length of the small intestine. Between segments 2-3, 5-6 and 8-9, two cm pieces of the intestine were removed for measurement of the circumference as described before (Nabuurs et al., 1993b).

Perfusion procedure

Fifteen minutes before perfusion started segments 2,4,6,8 and 10 were injected with 5 ml of PBS and the other segments were injected with 5 ml of the ETEC suspension in PBS. Simultaneously, the ten segments were perfused with the different fluids all perfusing an uninfected as well as the adjacent ETEC-infected segment.

Each segment was perfused with 64 ml of perfusion fluid over 8h, by injecting 2 ml of fluid every 15min. The non-absorbed fluid was able to drain freely into the drainage bottles. At the end of the experiment the fluid remaining in the segments was blown out into the drainage bottles (outflow). The piglets were killed by injection of 200 mg/kg bodyweight

sodiumpentobarbital. The segments were cut from the mesenterium and the length was measured (Nabuurs et al., 1993b).

Chemical analysis

Sodium and chloride concentrations of the perfusion fluids and the outflow were determined using an Electrolyte 4^+ analyser (Noval Biomedical, Waltham, USA). Osmolality was determined using a cryoscopic osmometer (Osmomat, Gonotec, Berlin, Germany).

Net absorption

Net fluid absorption was defined as the difference between the volume of inflow and the volume of outflow, divided by the surface area (length × circumference) of the segment. The net absorption of sodium and chloride was calculated from the volume and concentration in the perfusion fluid and the volume and concentration in the outflow, divided by the surface area of the segment (Nabuurs et al., 1994; Nabuurs et al., 1993b).

Statistics

The net absorption of fluid, sodium and chloride was determined as the mean ± standard error of the mean. Results of the first experiment were analysed using linear regression. Results from uninfected and ETEC-infected segments perfused with the same perfusion fluid were compared using the Student's *t* test, whereas comparisons between different perfusion fluids tested in the Latin Square design were made using one-way analysis of variance (ANOVA).

RESULTS

Increased osmolality resulted in a decreased net fluid absorption (Figure 5.1). A linear relationship between the osmolality of the perfusion fluids and the average net fluid absorption for both uninfected ($y=-1.9x+1162$) and ETEC-infected ($y=-2.5x+947$) segments was found (Figure 5.1). The slopes of the regression lines for uninfected and ETEC-infected segments were not statistically different ($P=0.39$), but differences between the elevations were ($P<0.0001$).

Increased sodium and chloride concentration and hence increased osmolality of the perfusion fluid resulted in significant increased sodium and chloride concentrations and osmolality of the outflow of both control and ETEC-infected segments (Table 5.2). There were no significant differences between sodium and chloride concentration and osmolality in the outflow of uninfected and corresponding ETEC-infected segments.

In Table 5.3 it is shown that net sodium and chloride absorption was significantly higher for uninfected segments compared to ETEC-infected segments. Furthermore it is shown that increase in sodium and chloride concentration of the perfusion fluid resulted generally in increased absorption of these ions, except for perfusion fluid C375, but the differences between the perfusion fluids used were not significant.

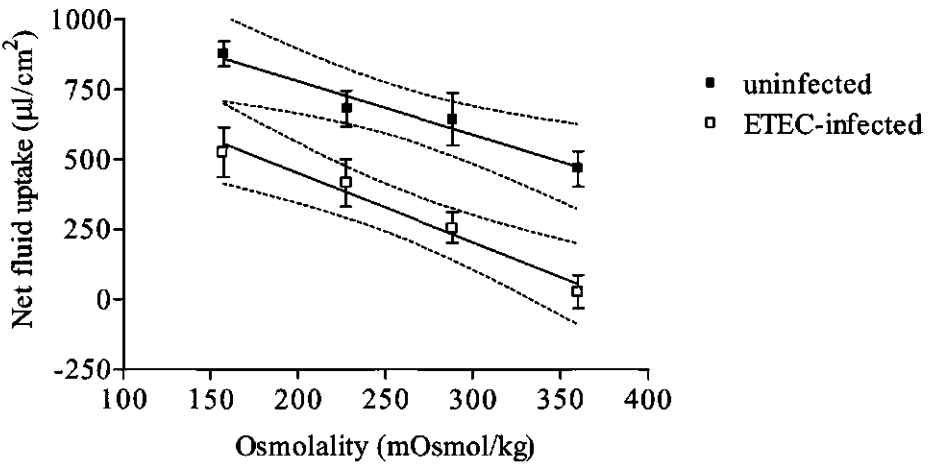

Figure 5.1 Average net fluid absorption (n=8, ± standard error of the mean) in uninfected and ETEC-infected segments perfused with solutions differing in sodium chloride concentrations (C150, C225, C300 and C375) resulting in osmolalities of 150-375 mOsmol/kg. Linear regression lines with 95% confidence intervals are shown.

Osmolality of 560 mOsmol/kg (C600) resulted in a net fluid secretion even in the uninfected segments (Figure 5.2A). Reducing osmolality beyond 150 mOsmol/kg did not result in a significant increase in net fluid absorption. ETEC infection resulted in a decrease in net fluid absorption of about 400 µl/cm^2 independent of the osmolality of the perfusion fluid used (Figures 5.1 and 5.2A). During perfusion with C0 net sodium secretion occurred, whereas near maximal fluid absorption was observed (Figure 5.2). At osmolalities higher than 150 mOsmol/kg (corresponding to 75 mM of sodium chloride) only a small increase in sodium absorption was seen. Osmolality of the outflow increased during perfusion with fluids of increasing osmolality (Figure 5.2C). In the range of 0-150 mOsmol/kg, osmolality in the outflow of uninfected segments was lower compared to ETEC-infected segments, whereas at higher osmolalities of the perfusion solution the osmolalities of the outflow were identical for uninfected and ETEC-infected segments.

Table 5.2 Average sodium concentration, chloride concentration and osmolality in the outflow after perfusion of uninfected and ETEC-infected segments with solutions C150-C375.

	Na (mM)		Cl (mM)		Osmolality (mOsmol/kg)	
	Uninfected	ETEC	Uninfected	ETEC	Uninfected	ETEC
C150	116±3	123±3	106±6	102±1	244±10	258±8
C225	132±1	132±1	117±3	113±2	267±2	274±4
C300	143±1	141±1	127±3	124±2	286±3	288±2
C375	156±2	151±2	141±5	132±4	309±3	302±2

Table 5.3 Average net sodium and chloride absorption after perfusion of uninfected and ETEC-infected segments with solutions C150-C375.

	Na (μmol/cm^2)		Cl (μmol/cm^2)	
	Uninfected	ETEC	Uninfected	ETEC
C150	68±4	27±11 [b]	70±4	35±9 [b]
C225	76±8	40±12 [a]	76±8	48±9 [a]
C300	100±15	46±8 [b]	99±14	53±8 [a]
C375	101±10	39±9 [c]	96±11	47±7 [b]

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ for ETEC-infected vs. uninfected.

Substitution of half of the sodium chloride with mannitol in perfusion fluids with total osmolalities of around 150 and 300 mOsmol/kg (solution C150 versus C150M and C300 versus C300M) resulted in major reductions in average net fluid absorption (Table 5.4) both in uninfected and ETEC-infected segments. Doubling the osmolality using sodium chloride (C150 versus C300) resulted in a decrease of net fluid absorption conform the data shown in Figure 5.1 and 5.2A, while doubling osmolality using mannitol (C150 versus C300M) resulted in a distinct larger decrease in net fluid absorption, respectively 901±124 versus 99±88 μl/cm^2 for uninfected and 568±96 versus –193±62 μl/cm^2 for ETEC-infected segments.

Average net fluid absorption was not statistically different (P=0.89) for WHO perfusion fluid and sodium chloride based perfusion fluid C300 in uninfected segments (Table 5.4). ETEC-infected segments perfused with the WHO perfusion fluid showed significant (P=0.04) higher net fluid absorption compared to segments perfused with sodium chloride based perfusion fluid C300, respectively 240±67 versus 44±33 μl/cm^2.

Table 5.4 Average net fluid absorption (n=4, ± standard error of the mean) in uninfected and ETEC-infected segments perfused with five perfusion fluids (C150, C150M, C300, C300M and WHO) differing in solute composition and/or osmolality.

Osmolality	Perfusion fluid	Uninfected	ETEC-infected
±150	C150	901±124	568±96
	C150M	628±134	348±92
±300	C300	536±231	44±33
	C300M	99±88	-193±62[a]
±300	WHO	490±201	240±67[ab]

[a] $P < 0.05$, versus C300
[b] $P < 0.01$, versus C300M

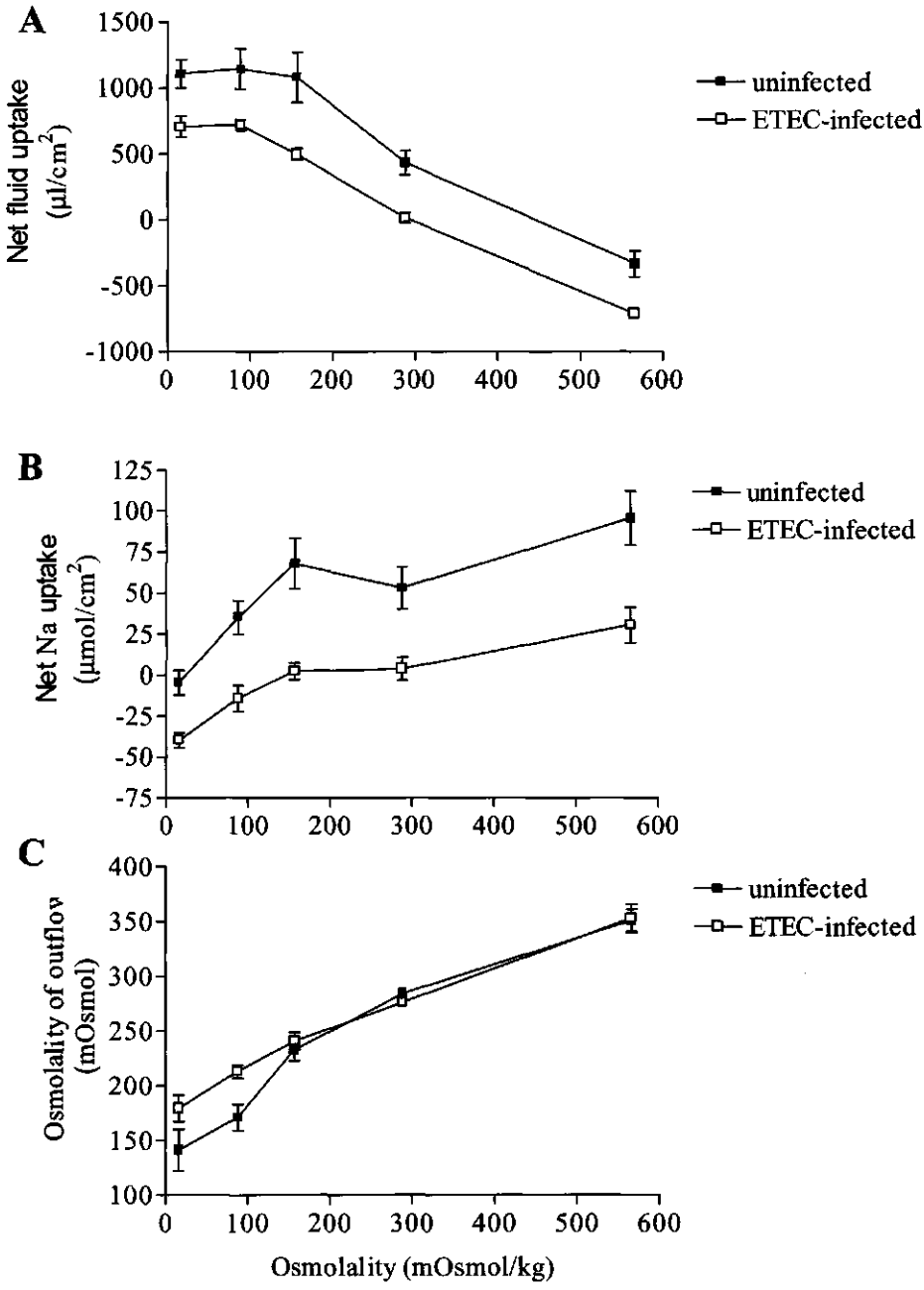

Figure 5.2 Results of perfusion of uninfected and ETEC-infected segments with solutions differing in sodium chloride concentrations (C0, C75, C150, C300 and C600) resulting in osmolalities of 0-600 mOsmol/kg (n=5, ± standard error of the mean). **A.** Average net fluid absorption. **B.** Average net sodium absorption. **C.** Osmolality of the outflow.

DISCUSSION

The SISP model enables us to study the effect of different perfusion solutions on net fluid absorption in uninfected and ETEC-infected small intestinal segments within one piglet. Studying the effect of osmolality on net fluid absorption, we observed a linear decrease in net fluid absorption when perfusion fluids of increasing osmolality were used. This corresponds well to data obtained from rat small intestine (Thillainayagam et al., 1993; Wall et al., 1997). A similar relationship was shown in ETEC-infected segments. Other workers also found a significant correlation between the osmolality of an ORS and the net fluid absorption in cholera toxin-induced secreting rat intestine (Cunha Ferreira et al., 1992; Pillai et al., 1994a).

Below 150 mOsmol/kg the inverse relation between water absorption and osmolality of the perfusion solution disappeared, as was also shown before in the human jejunum (Soergel et al., 1968). In this study we observed a high net fluid absorption at low osmolality despite low net sodium absorption or even a net sodium secretion . Net fluid absorption despite net sodium secretion in studies on secreting rat intestine was shown earlier (Cunha Ferreira et al., 1992; Pillai et al., 1994a; Wall et al., 1997). Most probably an extremely high osmotic gradient between villous interior and intestinal lumen was created. This could be a more critical factor than fluid absorption depending on solute (sodium) absorption. This hypothesis is supported by the decreased net fluid absorption at osmolalities higher than 150 mOsmol/kg despite more or less stable net sodium absorption observed in this study.

Although it is shown in this study and several others (Farthing, 1990; Hunt et al., 1992; Hunt et al., 1994; Pillai et al., 1994a; Thillainayagam et al., 1993; Thillainayagam et al., 1998) that low osmolality promotes net fluid absorption in normal and secreting intestine, we clearly showed that hypotonic solutions do not eliminate the decreased net fluid absorption due to ETEC infection. The difference between the net fluid absorption in uninfected and ETEC-infected segments is fairly independent of the osmolality of the perfusion solution (Figures 5.1 and 5.2). The sodium and chloride concentrations as well as the osmolality of the outflow of the ETEC-infected segments did not differ from that in the uninfected segments, reflecting the capacity of the tissue to react to the infection and maintain homeostasis by rapidly returning luminal osmolality to isotonicity. Maintaining luminal isotonicity therefore probably has a higher priority than keeping a positive fluid balance.

Substitution of sodium chloride with mannitol considerably decreased net fluid absorption both in uninfected and ETEC-infected segments. Similar findings were observed in secreting rat small intestine when a basic perfusion solution was supplemented with mannitol (Cunha Ferreira et al., 1992). Mannitol can not be actively absorbed and therefore will have a negative effect on fluid absorption, because the unabsorbed molecules contribute to luminal osmotic load. The significantly increased net fluid absorption in ETEC-infected segments perfused with WHO-ORS compared to a sodium chloride solution of similar osmolality was probably the result of the presence of a high glucose concentration in the WHO perfusion solution. Because of the ETEC infection neutral sodium chloride absorption is inhibited whereas the glucose-sodium co-transporter system remains intact resulting in fluid absorption.

In conclusion, we have shown that perfusion of uninfected piglet small intestinal segments with hypotonic solutions resulted in an increased net fluid absorption. Furthermore it was clearly shown that net fluid absorption was reduced in ETEC infection, but a similar inverse correlation between osmolality and net fluid absorption as in uninfected intestine was observed. During hypo- and hyperosmolar luminal conditions and during ETEC infection, maintaining a positive fluid and electrolyte balance appeared inferior to maintaining isotonic luminal conditions. It was confirmed that besides osmolality the ability of a solute (sodium chloride, mannitol, and glucose) to be absorbed and the mechanism by which it is absorbed

will determine to a great extent fluid absorption. Although hypotonicity of intestinal lumen by itself has no direct curative effect on ETEC infection, it can be beneficial in postweaning diarrhoea. The SISP model in early weaned piglets is useful to test anti-diarrhoea agents and ORSs on their capability to decrease the observed difference in net fluid absorption between uninfected and ETEC-infected segments.

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Chapter 6

Effect of processed and fermented soya bean on net absorption in piglet small intestine

Enterotoxic diarrhoea is a major problem in both human and veterinary medicine. Infection of enterotoxigenic *Escherichia coli* (ETEC) results in high fluid secretion and electrolyte losses in the small intestine. Minimising fluid and electrolyte losses by reducing secretion or stimulating absorption are of major importance in the treatment of ETEC diarrhoea. Tempe, a fermented soya bean product, was reported to be beneficial in terms of duration of diarrhoeal episodes and rehabilitation period when supplemented to the diet of malnourished children. Soya bean was processed into an autoclaved, a cooked and a fermented (tempe) product. The soya bean products were pre-digested and small intestinal segment perfusion (SISP) was used to study the effect of the products on net absorption. All three processed soya bean products appeared to protect against the fluid loss due to ETEC infection. Cooked soya bean and tempe showed highest protection. Net fluid absorption was highest for cooked soya bean followed by autoclaved soya bean and tempe. ETEC infection hardly affected net sodium and chloride absorption in segments perfused with soya bean tempe. In case of tempe significantly higher absorption of solutes other than sodium, chloride and potassium occurred. Processed soya bean products, particularly cooked soya bean and tempe, are beneficial in maintaining fluid balance during ETEC infection. Net fluid absorption was highest in the cooked soya bean product and was inversely correlated with the osmolality of the processed soya bean products. Tempe showed higher dry matter and total solute absorption than cooked soya bean and also sodium and chloride losses were reduced during ETEC infection. Therefore particularly tempe may be beneficial in case of postweaning diarrhoea in piglets and possibly in children as well.

Submitted for publication

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INTRODUCTION

Diarrhoea is one of the major causes of infant and childhood mortality in developing countries, with an estimated 1000 million episodes and 3.3 million deaths occurring each year among under-5-year-olds (Bern et al., 1992). The pathogens most strongly associated with acute diarrhoea in children are rotavirus, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC) (Bhan, 2000; Huilan et al., 1991). Problems with acute diarrhoea also frequently occur in pig husbandry. In pig husbandry ETEC is the main etiologic agent in both pre- and postweaning diarrhoea (Hampson, 1994).

Tempe is a traditional fermented food made from soaked and cooked soya beans inoculated with a mould, usually of the genus *Rhizopus* (Nout and Rombouts, 1990; Steinkraus, 1996). After fermentation has occurred, the soya beans 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, which hydrolyse soya bean constituents and contribute to the development of a desirable texture, flavour, and aroma of the product (Hachmeister and Fung, 1993). Enzymatic hydrolysis also may decrease or eliminate anti-nutritional constituents. Overall, the nutritional quality of the fermented product is improved (Karyadi et al., 1987). Tempe has been shown to inhibit *E. coli* infection in rabbits (Karmini et al., 1997; Karyadi et al., 1990) and was reported to be beneficial in terms of shortening diarrhoeal episodes and rehabilitation period when supplemented to the diet of malnourished children (Kalavi et al., 1996; Soenarto et al., 1997).

Early weaned piglets show large similarities in gastrointestinal (patho)physiology to young children (Miller and Ullrey, 1987; Moughan et al., 1992). The small intestinal segment perfusion (SISP) model (Nabuurs et al., 1993b) enables the perfusion of small intestinal segments within one piglet simultaneously in the absence and in the presence of ETEC. Against the background of reported beneficial effects of tempe in the control of diarrhoeal disease, we have used the SISP model to study the effect of processed and mould-fermented soya bean products on net absorption in uninfected and ETEC-infected piglet small intestine.

MATERIALS AND METHODS

Soya bean products

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater using accelerated acidification (Nout et al., 1987). Subsequently, the beans were washed with tapwater and cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), 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 (Oxoid, CM 59) for 7 days at 30°C and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone. After inoculation of the cooked soya beans with the sporangiospore suspension (1% v/w) the beans (450g) were packed into hard-plastic, perforated boxes (205×90×45 mm) and incubated at 30°C for 72h.

Raw, cooked and fermented soya beans were dried for 6h at 60°C and ground (Fritsch, type Pulverisette 14, Germany) using a 1.0 mm screen and stored at -20°C until use.

Pre-digestion

Pre-digestion was carried out as described earlier using α-amylase, pepsin, lipase and pancreatin (Kiers et al., 2000a). Autoclaved soya bean was prepared by autoclaving ground raw soya bean for 30 min at 121°C and was subsequently pre-digested as was done for raw, cooked and fermented soya bean. After pre-digestion the slurries were diluted to approximately 6.5% dry matter and kept at 4°C until use in the SISP test the next day.

Animals

Piglets (Yorkshire × (Large White × Landrace)) were weaned at three weeks of age. They were transported to the institute and fed a standard piglet feed. Water containing 60 mg/l colistinsulphate was applied *ad libitum*. About two weeks after weaning biopts from the duodenal mucosa were taken using a fiberscope (Olympus GIF XP10, Olympus, Hamburg, Germany) and receptor status was determined essentially as described before (Sellwood et al., 1975). Piglets that expressed receptors involved in binding of the ETEC strain were used in the experiments three weeks after weaning.

All experiments were conducted in accordance with the standards of the Ethic Committee of the Institute.

Small Intestinal Segment Perfusion (SISP)

The anaesthetic and surgical procedures used were essentially as described before (Nabuurs et al., 1993b). The animal was tranquillised with 2 mg/kg bodyweight azaperon. Anaesthesia was induced with halothanum and nitrous-oxide. The abdominal cavity was opened and the first intestinal segment was prepared approximately 75 cm caudal to the stomach. A small cranial tube (inflow) was placed and a wide tube (outflow) was placed 20 cm distal from the first. Caudal and adjacent to this first segment nine other segments were prepared in the same way. In this way the ten segments were situated between 9±2%–34±4% of the total length of the small intestine. Between segments 2-3, 5-6 and 8-9, 2 cm pieces of the intestine were removed for measurement of the circumference as described before (Nabuurs et al., 1993b).

The odd numbered segments were injected with 5 ml enterotoxigenic *Escherichia coli* (ETEC) (5×10^9 colony-forming-units (cfu) O149:K91:K88^{ac}, producing heat-labile and heat-stable toxin STb) and the even numbered segments with 5 ml phosphate-buffered saline (PBS), whereupon the segments were perfused during 8h (Nabuurs et al., 1993b).

Saline (supplemented with 0.1% glucose and 0.1% casamino acids), pre-digested raw soya bean (SR), pre-digested autoclaved soya bean (SA), pre-digested cooked soya bean (SC), and pre-digested soya bean tempe (ST) were tested in three experiments of four piglets. In each piglet, five pairs of segments (an ETEC-infected and an adjacent uninfected) were perfused using Latin square design for the four soya bean products with saline in the middle (segments 5 and 6). Each segment was perfused with 64 ml of product over 8h, by injecting 2 ml of product every 15 min. The non-absorbed material/fluid was able to drain freely into a drainage bottle. 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/kg bodyweight sodiumpentobarbital. The segments were cut from the mesenterium and the length was measured (Nabuurs et al., 1993b). Samples of 1 cm^2 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 0044-17-9) supplemented with 5% of

defibrinated sheep blood. Plates were incubated for 18-24h at 37°C and haemolytic colonies were counted.

Dry matter content, sodium, potassium, chloride and osmolality analysis

Dry matter content of the products as well as the outflows was determined by drying aliquots for 24h at 80°C in triplicate. Sodium, potassium and chloride concentrations of the products and the outflows were determined using an Electrolyte 4+ analyser (Noval Biomedical, Waltham, USA). Osmolality was determined using a cryoscopic osmometer (Osmomat, Gonotec, Berlin, Germany).

Calculations

Net fluid, dry matter, sodium, potassium, chloride and total solute absorption was calculated from the difference between the volume and concentration of inflow and outflow divided by the surface area (length × circumference) of the segment (Nabuurs et al., 1994; Nabuurs et al., 1993b).

Statistics

The net absorption of fluid, dry matter, sodium, potassium, chloride and total solutes was determined as the mean ± standard error of the mean. Results were analysed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test when overall $p<0.05$. Comparisons in case of results obtained from uninfected vs. ETEC-infected segments for one product were made by paired t-tests.

RESULTS

Product characteristics

Characteristics of the pre-digested processed and fermented soya bean products are shown in Table 6.1. All characteristics were very similar for raw (SR) and autoclaved (SA) soya bean products. Potassium content in SR and SA was much higher compared to cooked (SC) and fermented (ST) soya bean. Osmolality was lowest for SC and almost double that value in ST.

Table 6.1 Characteristics of pre-digested soya bean products. Average of three pre-digestions ± standard deviations are given.

Product	pH	Dry matter (% w/w)	Sodium (mM)	Potassium (mM)	Chloride (mM)	Osmolality (mOsmol/kg)
SR[#]	6.3 ± 0.0	6.50 ± 0.23	48 ± 1	35.9 ± 5.8	57 ± 3	200 ± 11
SA	6.2 ± 0.0	6.54 ± 0.12	46 ± 3	36.9 ± 2.8	56 ± 2	198 ± 9
SC	6.1 ± 0.2	6.39 ± 0.14	44 ± 3	10.4 ± 1.1	41 ± 4	152 ± 12
ST	6.1 ± 0.1	6.90 ± 0.02	63 ± 3	11.7 ± 1.4	62 ± 4	294 ± 11

[#] SR= pre-digested raw soya bean, SA= pre-digested autoclaved soya bean, SC= pre-digested cooked soya bean, ST= pre-digested soya bean tempe

Net fluid absorption

Net fluid absorption was highest for SC followed by SA and ST, whereas SR showed the lowest fluid absorption (Table 6.2). An inverse linear relationship was found between average net fluid absorption and osmolality of the pre-digested processed soya bean products (SA, SC and ST) both for the uninfected ($y=-1.27x+844$, $r^2=0.998$) and the ETEC-infected ($y=-1.33x+808$, $r^2=0.931$) situation. Perfusion of SR resulted in significant lower net fluid absorption values compared to SA despite equal characteristics (Table 6.1) including osmolality. ETEC infection resulted in a decreased net fluid absorption of 260±23 µl/cm^2 for saline. Reduction in average net fluid absorption (delta) was much less pronounced in soya bean products (Table 6.2). All soya bean products appeared to protect against the fluid loss upon ETEC infection (Figure 6.1). Mean protection against fluid loss was considerably higher for SC and ST when compared to SR and SA.

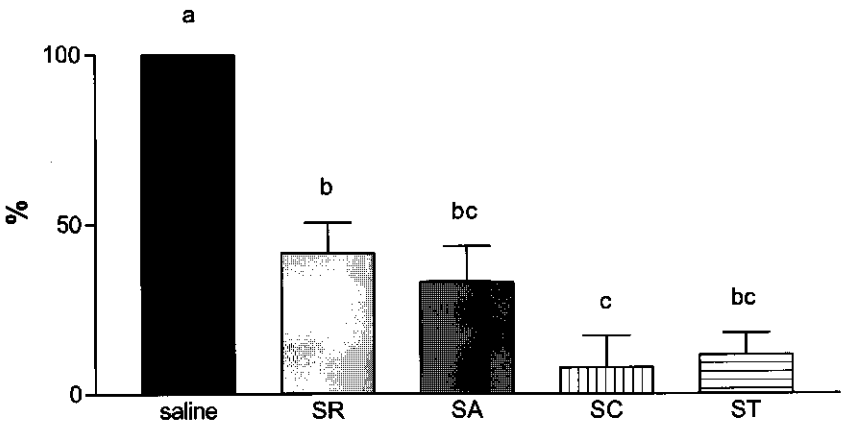

Figure 6.1 Fluid losses after perfusion with the pre-digested soya bean products relative to saline. Columns with different superscripts are significantly different. For labels/abbreviations see Table 6.2.

Net dry matter absorption

Net dry matter absorption was higher from soya bean products compared to saline and was significantly higher for processed soya bean compared to SR (Table 6.2). No significant differences were found in net dry matter absorption between the three processed soya bean products, although ST was higher than SA and SC. Net dry matter absorption was not significantly different between uninfected and ETEC-infected segments for all processed soya bean products, but was for saline and SR.

Net sodium, chloride and potassium absorption

Perfusion of SR resulted in net secretion of sodium in both uninfected and ETEC-infected segments (Table 6.2). Net sodium absorption was similar for SA, SC and ST in uninfected segments and was significantly reduced in ETEC-infected segments, although the reduction in ST was less severe.

Table 6.2 Average net absorption of fluid, dry matter, sodium, chloride and potassium. Average ± standard error of mean is shown both for uninfected and ETEC-infected segments.[#]

Product	Fluid ($\mu l/cm^2$)			Dry matter (mg/cm^2)				Sodium ($\mu mol/cm^2$)			Chloride ($\mu mol/cm^2$)			Potassium ($\mu mol/cm^2$)		
	uninf.	*+ETEC*		*Delta*	*uninf.*	*+ETEC*		*uninf.*	*+ETEC*		*uninf.*	*+ETEC*		*uninf.*	*+ETEC*	
Saline[##]	469±33^{a}	209±42^{a}	***	260±23^{a}	1.5±0.8^{a}	-4.1±1.0^{a}	***	74±5	41±6^{a}	***	66±5^{a}	47±6^{a}	***	-2.6±0.2	-5.6±0.4	***
SR[###]	340±11^{b}	230±22^{a}	***	109±23^{b}	7.6±0.8^{b}	1.2±1.4^{a}	**	-5±2	-27±2^{b}	***	7±1^{b}	0±1^{b}	*	20.3±1.1	16.0±0.8	***
SA	581±47^{c}	510±39^{b}	**	71±19^{bc}	13.2±1.3^{c}	12.9±1.8^{b}	ns	21±3	1±3^{c}	***	33±4^{c}	23±3^{c}	***	22.3±1.8	22.4±2.1	ns
SC	646±52^{c}	619±47^{c}	ns	27±23^{c}	13.3±1.5^{c}	11.5±1.5^{b}	ns	21±2	12±3^{d}	**	22±3^{d}	17±2^{c}	**	4.4±0.5	3.4±0.4	**
ST	465±52^{a}	422±54^{d}	ns	43±20^{bc}	16.1±1.7^{c}	15.0±2.0^{b}	ns	24±3	18±4^{d}	*	29±4^{c}	24±4^{c}	**	3.4±0.5	2.5±0.5	**

[#] Figures with different superscripts within each column are significantly different. *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$ and ns = not significant for uninf. vs. ETEC-infected.
[##] Saline= physiological salt solution supplemented with 0.1% glucose and 0.1% casamino acids.
[###] For abbreviations see Table 6.1.

Net total solute absorption

Perfusion of saline resulted in high net total solute absorption (Figure 6.2, upper panel). The sum of the net absorption of sodium, chloride and potassium almost equalled the net total solute absorption for saline (Figure 6.2, lower panel). SR showed net secretion of solutes in uninfected as well as ETEC-infected segments. Of all soya bean products ST showed highest net total solute absorption. It is clearly shown that for all tested products except for ST a net secretion of solutes occurred when corrected for sodium, chloride and potassium absorption (Figure 6.2, lower panel). In addition to sodium, chloride and potassium other solutes were absorbed in uninfected and ETEC-infected segments perfused with ST.

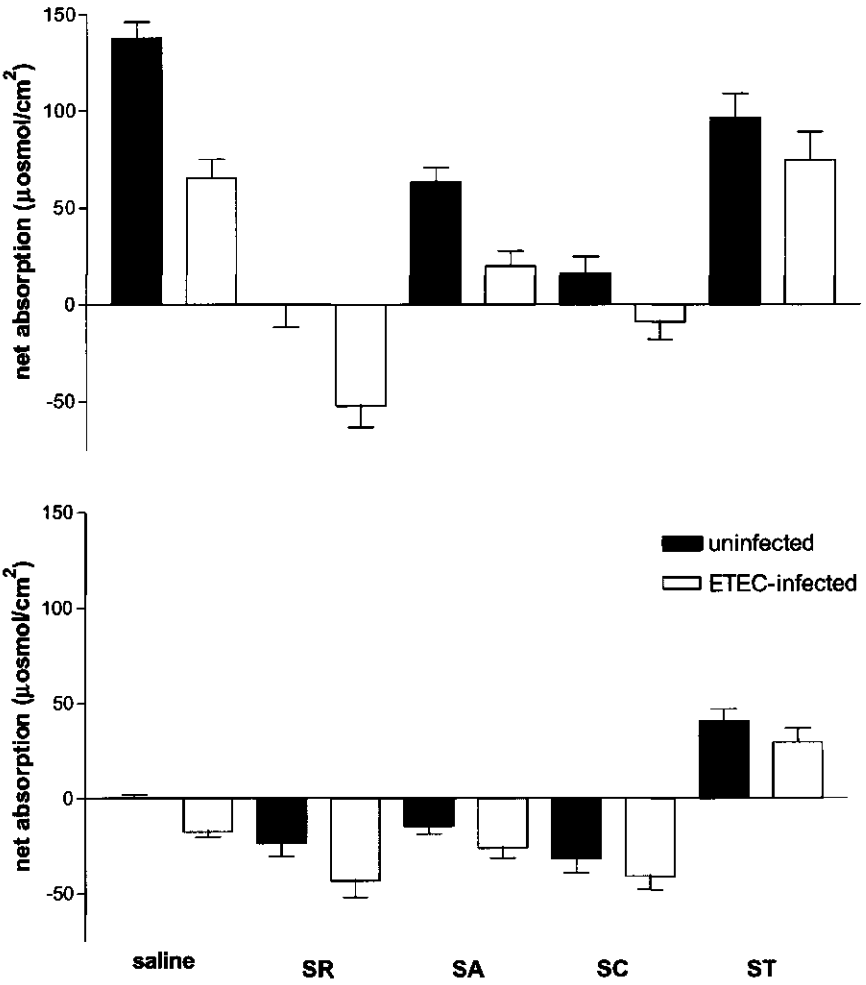

Figure 6.2 Average net absorption of solutes. Top panel: Total solutes. Lower panel: Total solutes minus sodium, chloride and potassium. For labels/abbreviations see Table 6.2.

Determination of ETEC attached to the intestinal mucosa

Bacteriological analysis of the mucosa from ETEC-infected segments showed high numbers of ETEC for all soya bean products (Table 6.3). Segments perfused with saline showed significantly lower ETEC numbers.

Table 6.3 Number of ETEC attached to the infected segmental wall after perfusion with saline and pre-digested soya bean products. Results are shown as $\log_{10}$ (cfu/cm^2) ± standard error of the mean.[#]

Product	ETEC $\log_{10}$ (cfu/cm^2)
Saline[##]	6.55±0.34[a]
SR	8.25±0.18[b]
SA	8.62±0.22[b]
SC	8.90±0.19[b]
ST	8.73±0.23[b]

[#] Figures with different superscripts are significantly different
[##] For abbreviations see Table 6.2

DISCUSSION

Tempe is a traditional fermented food made from soaked and cooked soya beans inoculated with a mould, usually of the genus *Rhizopus*. During the tempe making process the soya beans are soaked and cooked. Considerable leaching of soluble dry matter occurs (Kiers et al., 2000a) which explains the low potassium content of the cooked and fermented soya bean 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 (De Reu, 1995; Kiers et al., 2000a) and the addition of excess HCl and $NaHCO_3$ during the pre-digestion to adjust pH for the different enzymatic degradation steps (Kiers et al., 2000a).

High dry matter absorption during perfusion with tempe might reflect an improved nutrient availability after fermentation as was suggested earlier (Kiers et al., 2000a). It was probably not the result of differences in osmolality and sodium concentration between cooked soya bean and tempe because firstly, low osmolality does not affect the absorption rates of total nitrogen and carbohydrate from nutrient solutions (Pfeiffer et al., 1998) and secondly sodium concentration of enteral diets do not influence absorption of macronutrients and of total energy in miniature pigs (Ehrlein et al., 1999). When total net solute absorption was corrected for sodium, chloride and potassium only tempe showed a positive balance, probably reflecting the uptake of easily accessible nutrients and/or minerals (Kiers et al., 2000a; Macfarlane et al., 1990; Mital and Garg, 1990).

The significant inverse correlation between the processed soya bean product osmolality and net fluid absorption described in this study was shown earlier for sodium chloride based perfusion solutions (Chapter 5) and oral rehydration solutions in which the glucose content had been partially replaced by amino acids or food supplements (Pillai et al., 1994a; Pillai et al., 1994b).

The difference in the net fluid absorption in uninfected versus ETEC-infected segments (delta) was higher for saline compared to the different soya bean products. The observed different protective effects of the soya bean products were not caused by their different osmolalities, since we showed earlier that osmolality of a perfusion solution did not affect the degree of reduction in net fluid absorption due to ETEC infection (Chapter 5). Especially

cooked soya beans and tempe minimised the reduction in net fluid absorption in ETEC-infected segments. Possible explanations are outlined below.

The presence of one or more antibacterial substances in tempe has been described although these were reported to act against Gram-positive bacteria only (Chapter 4; Anonymous, 1969; Wang et al., 1972; Wang et al., 1969). Possible inhibition of ETEC adhesion to specific receptors has been shown for a range of glucosides and glucosamine (Payne, 1994), lactoferrin (Giugliano et al., 1995) and component(s) produced by *Lactobacillus* spp. (Blomberg et al., 1993; Ouwehand and Conway, 1996). Compounds in the soya bean products might interfere with the attachment of the ETEC to the enterocytes, as we showed for tempe before (Chapter 4). Furthermore, specific proteolytic activity in the intestine has been shown to inactivate K88 ETEC receptors and consequently protect against ETEC-induced diarrhoea (Chandler and Mynott, 1998; Mynott et al., 1997; Mynott et al., 1996). The pre-digested tempe product could show proteolytic activity (fermentation derived proteases) during perfusion. However, since high numbers of ETEC were detected after sampling the segmental wall after perfusion, probably no antibacterial activity and no conclusive interference with the adhesion of ETEC occurred during perfusion with the soya bean products.

Possibly the presence of insoluble compounds like fibres could explain at least part of the observed protective effect of the soya bean products including SR by affecting viscosity and/or transit time as was shown earlier (Ehrlein and Stockmann, 1998; Go et al., 1994). However, in our study we did not observe any differences in viscosity of the soya bean products (data not shown). On the other hand, soluble fibre has been shown to enhance intestinal water and electrolyte absorption in normal and secreting rat small intestine (Turvill et al., 2000; Wapnir et al., 1996; Wapnir et al., 1998; Wingertzahn et al., 1999) and has shown an anti-secretory effect on water, sodium, and chloride induced secretion by dibutyryl cyclic AMP in rat small intestine as well (Rabbani et al., 1991). Inhibition of intestinal secretion was shown for proanthocyanidins (Hor et al., 1995) and a factor purified from boiled rice (Macleod et al., 1995; Mathews et al., 1999) also. Therefore, the presence of (in)soluble fibres, stimulation of fluid absorption following coupled sodium-solute uptake, and the existence of compounds able to inhibit secretion or a combination of these factors could explain the protective effect of the processed soya bean products.

Beneficial effects of tempe in case of disease prevention and treatment, principally in case of diarrhoeal diseases and nutritional impact in Indonesian children, have been reported (Karyadi and Lukito, 1996; Karyadi and Lukito, 2000; Karyadi et al., 1990). In our study we showed that soya bean tempe did protect against ETEC-induced fluid losses but this was also shown for cooked soya bean. In tempe also sodium losses were less severe and higher dry matter and solute absorption was shown compared to cooked soya bean. Because of its protective effects during ETEC infection and its improved digestibility/nutrient availability, tempe may be beneficial in case of postweaning diarrhoea in piglets and in young children, where problems associated with enterotoxic diarrhoea and malnutrition are severe.

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Chapter 7

A high molecular weight fraction of tempe protects against fluid losses in ETEC-infected piglet small intestine

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhoea in young children and piglets. ETEC induced hypersecretion by the intestinal mucosa can cause rapid dehydration and death. Tempe, a fungal fermented soya bean product, protected against excessive fluid losses induced by ETEC infection in small intestinal segments of piglets. The protective effect of pre-digested tempe was compared to the effect of its constitutive fractions. Soya bean was processed and fermented using *Rhizopus microsporus*. After pre-digestion the product was centrifuged and the supernatant was separated by ultrafiltration using a 5kDa membrane. Small intestinal segment perfusion (SISP) was used to study the effect of the fractions on net absorption in uninfected and ETEC-infected segments. The presence of the insoluble material appeared to account for a considerable part of the protective effect of the pre-digested tempe product. However, the soluble fraction alone still showed 50% less fluid loss compared to saline. Observed protective effect was exclusively attributable to the high-molecular-weight (> 5kDa) fraction. In addition it was shown that pre-digestion was not a prerequisite for tempe to exert its protective effect. Tempe contains a high-molecular-weight fraction (> 5kDa) which protects against fluid losses induced by ETEC. In addition to its function as an easily digestible protein food, tempe could therefore play a role in controlling ETEC induced diarrhoea.

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INTRODUCTION

The pathogens most strongly associated with acute diarrhoea are rotavirus and enterotoxigenic *Escherichia coli* (ETEC) (Hampson, 1994; Huilan et al., 1991; Nabuurs et al., 1993c). Diarrhoeal diseases caused by ETEC remain an important health problem in children and young animals (Gaastra and Svennerholm, 1996; Huilan et al., 1991; Nagy and Fekete, 1999). Treatment options for acute gastro-enteritis mainly rely on oral rehydration solutions (ORSs), although the volume, frequency or duration of diarrhoea are not reduced using conventional ORSs (Bhan, 2000; Mahalanabis, 1996). Therefore anti-diarrhoea drugs and antibiotics are widely used (Mahalanabis, 1996).

Tempe is a traditional fermented food made from soaked and cooked soya beans inoculated with a mould, usually of the genus *Rhizopus* (Nout and Rombouts, 1990; Steinkraus, 1996). Tempe has been reported to contain an antibacterial substance (Wang et al., 1969) and has been shown to inhibit *E. coli* infection in rabbits (Karmini et al., 1997; Karyadi et al., 1990). Tempe was reported to be beneficial in terms of shorter duration of diarrhoeal episodes as well as rehabilitation periods when supplemented to the diet of malnourished children (Kalavi et al., 1996; Soenarto et al., 1997). Tempe, being a nutritious and easily digestible soya bean food (Kiers et al., 2000a; Nout and Rombouts, 1990; Steinkraus, 1996), may therefore also have beneficial effects in diarrhoea control.

Early weaned piglets show large similarities in gastrointestinal (patho)physiology to young children (Miller and Ullrey, 1987; Moughan et al., 1992). The use of piglets in studying ETEC infections not only holds promise as a model for ETEC infection in children, but also acute infectious diarrhoea associated with ETEC is of major concern in pig industry (Hampson, 1994; Nabuurs, 1998; Nagy and Fekete, 1999). The small intestinal segment perfusion (SISP) model (Nabuurs et al., 1993b) enables the perfusion of multiple small intestinal segments within one piglet and can therefore be used to study the effect of different products on net absorption of fluid and electrolytes simultaneously in the absence and in the presence of ETEC.

Perfusion of small intestinal segments of piglets with a pre-digested tempe product prevented against ETEC induced fluid losses (Chapter 6). In order to gain insight into the factor(s) responsible for the protective effect we attempted to determine the responsible fraction from the complex pre-digested tempe product.

MATERIAL AND METHODS

Tempe preparation

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater using accelerated acidification (Nout et al., 1987). Subsequently, the beans were washed with tapwater and cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), 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 (Oxoid, CM 59) for 7 days at 30°C and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone. After inoculation of the cooked soya beans with the sporangiospore suspension (1% v/w) the beans (450g) were packed into hard-plastic, perforated boxes (205×90×45 mm) and incubated at 30°C for 72h. The tempe was crumbled, dried for 6h at 60°C and ground (Fritsch, type Pulverisette 14, Germany) using a 1.0 mm screen and stored at -20°C until use.

Pre-digestion

Pre-digestion was carried out as described earlier using α-amylase, pepsin, lipase and pancreatin (Kiers et al., 2000a). After pre-digestion the suspension was diluted with distilled water to approximately 6.5% dry matter.

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, Fluka, 22140) to remove residual coarse particles and finally filtered through a 0.22 µm filter (Millipore, Steritop SCGPT05RE).

Ultrafiltration

Supernatant of pre-digested tempe was fractionated using ultrafiltration with a spiral wound membrane with molecular weight cut-off of 5kDa (Koch, S2K328) against distilled water. In order to obtain fractions of the same volume as the volume of supernatant which was ultrafiltrated, the volumes of the permeate and retentate were adjusted using rotary evaporation at 40°C.

Analyses

Dry matter content was determined by drying aliquots until constant weight and nitrogen content was measured using a NA2100 nitrogen analyser (Interscience). Sodium, potassium and chloride concentrations were determined using an Electrolyte 4+ analyser (Noval Biomedical, Waltham, USA). Osmolality was determined using a cryoscopic osmometer (Osmomat, Gonotec, Berlin, Germany).

Gel permeation chromatography (GPC) was performed on a LC-10Ai HPLC (Shimadzu) equipped with a Superdex Peptide column (Pharmacia Biotech 17-5003-01) 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 7000 to 200Da.

High performance size exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300×75 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-rad Labs) in combination with a TSK guard column (40×6 mm) and elution at 30°C with 0.2M $NaNO_3$ at 0.8 ml/min. The eluate was monitored using a refractive index detector. Calibration was performed using dextrans ranging from 500kDa to 180Da.

Animals

Piglets (Yorkshire × (Large White × Landrace)) were weaned at three weeks of age. Biopts from the duodenal mucosa were taken and receptor status was determined as described before (Sellwood et al., 1975). Piglets that expressed receptors involved in binding of the ETEC strain were used in the experiments three weeks after weaning.

All experiments were conducted in accordance with the standards of the Ethic Committee of the Institute.

Small Intestinal Segment Perfusion (SISP)

Piglets were tranquillised with 2 mg/kg bodyweight azaperon. Anaesthesia was induced with isoflurane (Forene, Abbott Lab Ltd, Queensborough, UK) and nitrous-oxide. The surgical and

perfusion procedures used were essentially as described before (Chapters 5 and 6; Nabuurs et al., 1994; Nabuurs et al., 1993b). Ten segments were situated between 35.1±3.4–65.4±5.6% (average ± standard deviation) of the total length of the small intestine. Fifteen minutes before the perfusion started, the odd numbered segments were injected with 5 ml enterotoxigenic *Escherichia coli* (ETEC) ($5x10^9$ colony-forming-units (cfu) O149:K91:K88^{ac}, producing heat-labile and heat-stable toxin STb) and the even numbered segments with 5 ml phosphate-buffered saline (PBS). In each piglet, pairs of segments (an uninfected and an adjacent ETEC-infected) were perfused with saline (supplemented with 0.1% glucose and 0.1% casamino acids) and the tempe products, using Latin square design. Each segment was perfused with 64 ml of product over 8h, by injecting 2 ml of product every 15 min. The outflow of each segment was able to drain freely into the corresponding drainage bottle. At the end of the experiment the product remaining in the segments was blown out into the drainage bottles. The piglets were killed by injection of 200 mg/kg bodyweight sodiumpentobarbital and the segments were cut from the mesenterium and the length was measured. Net fluid, sodium, potassium, chloride and total solutes absorption was calculated from the difference between the volume and concentration of inflow and outflow divided by the surface area (length × circumference) of the segment.

Three experiments were carried out. In experiment 1 the total pre-digested tempe was tested against its supernatant in four piglets. In experiment 2 the supernatant of the pre-digested tempe was tested against the permeate and retentate obtained after ultrafiltration in nine piglets. Finally, the supernatant of pre-digested tempe was tested against the supernatant of undigested tempe in four piglets in experiment 3.

Brush border adhesion

Brush borders expressing the receptors involved in binding of the ETEC were isolated from early weaned pigs as described before (Sellwood et al., 1975). Using a Bürker-Türk counting chamber the brush border suspension was diluted to 10^6 brush borders/ml. Products (25µl) were mixed with 25 µl ETEC suspension and incubated at room temperature with continuous gentle shaking. After 30 min 50 µl of brush border suspension was added and incubated at room temperature with continuous gentle shaking for 20 min. Phase contrast microscopy was used to count the number of ETEC cells attached to one brush border. At least 15 brush borders were examined.

Statistics

The net absorption of fluid, sodium, potassium, chloride and total solutes was determined as the mean ± standard error of the mean. Comparisons between different products tested in the Latin Square design were made using one way analysis of variance (ANOVA) and when overall $P<0.05$ Newman-Keuls post test was used.

RESULTS

Total pre-digested product vs. supernatant (experiment 1)

In experiment 1 the difference between average net fluid absorption in uninfected and ETEC-infected segments (delta) perfused with saline amounted to 534±128 $\mu l/cm^2$ and was significantly higher compared with the total pre-digested tempe (75±81 $\mu l/cm^2$) and its supernatant (282±111 $\mu l/cm^2$). The difference in average net fluid loss between the two soya bean products was substantial, but not statistically significant.

Ultrafiltration of the supernatant of pre-digested tempe

Figure 7.1 shows the chromatograms of HPSEC analysis of the fractions obtained after ultrafiltration of the supernatant of pre-digested tempe. Ultrafiltration of the supernatant of tempe resulted in one fraction containing only low-molecular-weight compounds (permeate) and a second fraction containing all compounds > 5kDa and some low-molecular-weight compounds (retentate). Similar chromatograms were observed for GPC protein/peptide analysis and areas under the curve were calculated (Table 7.1). Around 50% of the total dry matter in the tempe supernatant consisted of protein compounds (factor 6,25 × N) of which the bulk had a molecular weight < 5kDa. When the data of the two fractions expressed in Table 7.1 were added they equal the composition of the supernatant which indicated that material losses due to ultrafiltration and rotary evaporation were negligible.

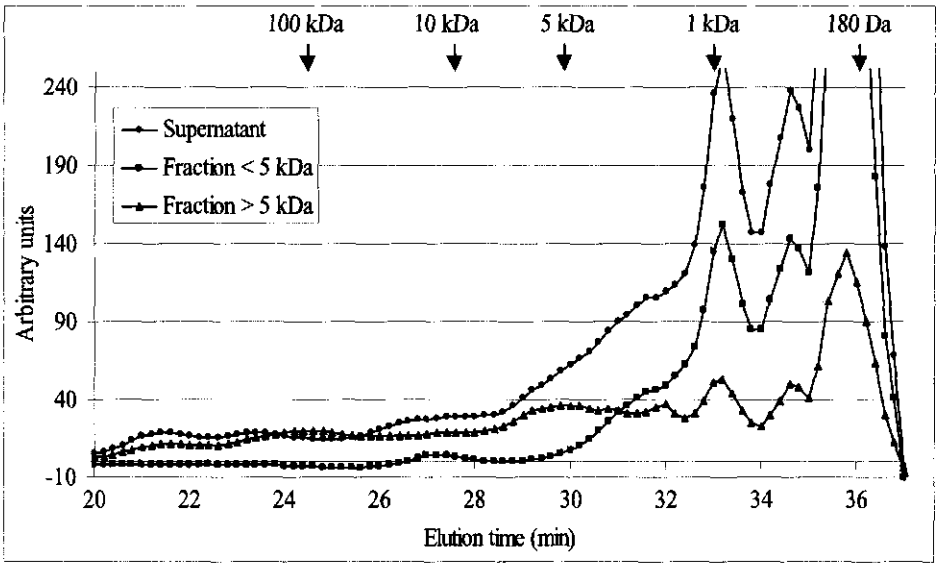

Figure 7.1 HPSEC elution pattern of the supernatant of pre-digested tempe and the fractions obtained after ultrafiltration. Molecular weights of several dextran standards are shown at the top of the figure.

Average net absorption from fractions obtained with ultrafiltration (experiment 2)

Average net absorption of fluid, electrolytes and total solutes in experiment 2 is shown in Table 7.2. Perfusion of uninfected segments with products of low osmolality (Table 7.1) resulted in the highest average net fluid absorption (Table 7.2). The difference between average net fluid absorption in uninfected and ETEC-infected segments perfused with saline amounted to 355±69 $\mu l/cm^2$. Although average net absorption of sodium, chloride, potassium and total solutes in uninfected segments was only slightly lower for the permeate compared to the supernatant, the values for ETEC-infected segments were markedly reduced. Figure 7.2 shows the fluid losses relative to saline. Perfusion with tempe supernatant resulted in a significant decrease in net fluid loss compared to saline. The permeate containing only components < 5kDa did not decrease net fluid loss whereas the retentate containing high-molecular-weight components showed equal protection compared to the total supernatant and showed a significant reduction in net fluid loss compared to saline.

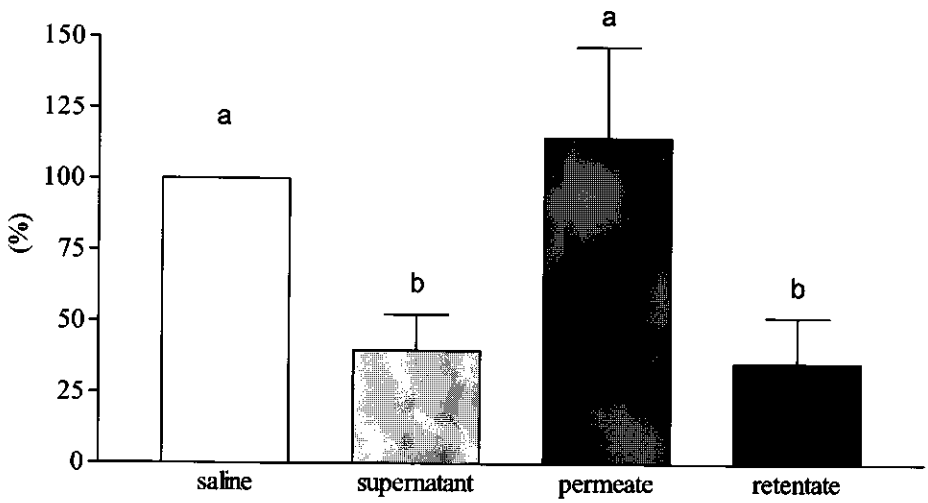

Figure 7.2 Fluid losses relative to saline after perfusion with the supernatant of pre-digested tempe and the fractions obtained after ultrafiltration. Bars marked with different letters are significantly different ($P < 0.05$).

Influence on ETEC adhesion to brush borders

Brush border adhesion inhibition was shown for the supernatant as well as for the permeate and the retentate. Inhibition amounted to approximately 50% and was not different for the three products tested.

Table 7.1 *Dry matter, nitrogenous compounds, sodium, chloride and potassium content and osmolality of the pre-digested supernatant and the two fractions obtained after ultrafiltration.*

	Dry matter (%)	Nitrogenous compounds (%)			Sodium (mM)	Chloride (mM)	Potassium (mM)	Osmolality (mOsmol/kg)
		Total	< 5kDa	> 5kDa				
Tempe supernatant	2.80	0.25	0.24	0.01	52±4	50±6	9.1±1.1	231±24
Permeate (< 5kDa)	1.94	0.16	0.16	0.00	40±8	41±7	6.7±1.2	169±34
Retentate (> 5kDa)	0.89	0.07	0.06	0.01	12±7	11±5	2.4±1.2	55±28

Table 7.2 Average net absorption of fluid, sodium, chloride, potassium and solutes (n=9).

	Fluid ($\mu l/cm^2$)		Sodium ($\mu mol/cm^2$)		Chloride ($\mu mol/cm^2$)		Potassium ($\mu mol/cm^2$)		Solutes ($\mu Osmol/cm^2$)			
									Total		Corrected*	
	uninfect	ETEC	uninfect	ETEC	uninfect	ETEC	uninfect	ETEC	uninfect	ETEC	uninfect	ETEC
Saline	559±79	204±57	89±16	41±7	92±16	49±7	-2.0±0.4	-4.8±0.4	234±26	95±16	-6±3	-15±3
Tempe supernatant	757±51	642±50	39±5	24±5	46±5	35±5	4.2±0.7	2.5±0.6	185±11	143±9	79±6	65±5
Permeate (< 5kDa)	882±76	597±39	36±6	5±4	41±6	19±4	4.0±0.7	0.2±0.5	164±21	75±12	65±8	40±5
Retentate (> 5kDa)	967±61	850±57	15±2	3±2	16±2	8±1	1.5±0.2	0.1±0.2	46±8	16±6	15±4	6±3

* Total solutes absorption corrected for sodium, chloride and potassium absorption

Table 7.3 Nitrogenous compounds and osmolality of the supernatant of undigested and pre-digested tempe.

	Nitrogenous compounds (%)			Osmolality (mOsmol/kg)
	Total	< 5kDa	> 5kDa	
Undigested	0.07	0.06	0.01	67±1
Pre-digested	0.20	0.20	0.01	217±1

Pre-digested vs. undigested tempe supernatant (experiment 3)

Pre-digestion of tempe did not result in liberation of soluble carbohydrate polymers > ±8.5kDa, whereas significant levels of low-molecular-weight compounds were formed (Figure 7.3). Pre-digestion resulted in a marked increase in nitrogenous compounds (of low molecular weight) and osmolality (Table 7.3).

In experiment 3 the difference between average net fluid absorption in uninfected and ETEC-infected segments perfused with saline amounted to 393±64 µl/cm^2. Fluid losses due to ETEC infection for undigested and pre-digested tempe supernatant were 211±72 µl/cm^2 and 214±83 µl/cm^2 respectively.

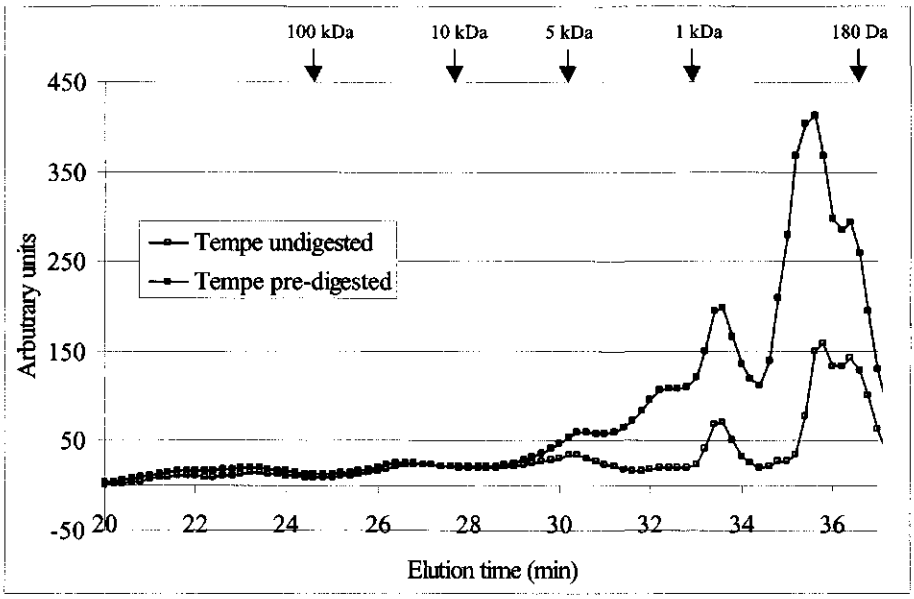

Figure 7.3 HPSEC elution pattern of the supernatant of undigested and pre-digested tempe. Molecular weights of several dextran standards are shown at the top of the figure.

DISCUSSION

Current studies extend our earlier work on the protective effect of processed and fermented soya bean on ETEC-induced fluid loss during perfusion of piglet small intestine. These earlier findings showed that tempe enhanced dry matter and total solute absorption and reduced ETEC-induced fluid and electrolyte losses (Chapter 6).

The protective effect of pre-digested tempe on ETEC-induced fluid loss appeared to be determined to a considerable extent by the presence of the insoluble matrix. The presence of insoluble material could probably modify fluid absorption as was suggested earlier for viscosity-enhancing agents (Go et al., 1994; Wapnir et al., 1997b). Whereas the total product reduced the net fluid loss by about 85% (this and previous study described in Chapter 6), the supernatant of pre-digested tempe alone still reduced the net fluid loss by almost 50% compared to saline.

In general, protection against ETEC-induced fluid loss is related to either interference with the pathogenesis of the ETEC infection resulting in a reduction in secretion or stimulation of absorption independent from the infection. Three possibilities whereby tempe could interfere with ETEC pathogenesis can be considered. First, since the tempe supernatant and its fractions (permeate and retentate) all reduced brush border adhesion *in vitro* by 50%, probably the observed protective effect of the supernatant and the retentate was not due to interference with ETEC adhesion. Secondly, proteases such as bromelain have been shown to degrade intestinal receptors in the gut (Chandler and Mynott, 1998; Mynott et al., 1997; Mynott et al., 1996), and since α-galactosidase and protease activity was found in the supernatant and in the retentate (> 5kDa) but not in the permeate (< 5kDa) (data not shown) this might explain the observed protective effects. Finally, components in the tempe extract could interfere with the (binding of) enterotoxins as was shown for certain toxin receptor analogues (Takeda et al., 1999) or with the cascade of calcium-mediated intracellular events triggered by enterotoxins leading to an increased chloride secretion and inhibited neutral sodium chloride absorption. Intestinal secretion has been shown to be inhibited by several plant extracts, mainly polyphenolic compounds (Hor et al., 1995; Meerveld et al., 1999), and a factor purified from boiled rice has been shown to block chloride channels (Mathews et al., 1999).

Tempe components could also exert a pro-absorptive activity. First, this can be mediated through stimulated sodium-solute co-transport which is the basis for traditional oral rehydration therapy (Chapter 5; Farthing, 1994). In our previous study we have shown a net positive balance in the uptake of total solutes even when corrected for sodium, potassium and chloride, which suggested the uptake of nutrients (Chapter 6). In the present study a net uptake of total solutes was observed both in the tempe supernatant as well as in the two fractions. Probably this reflects the uptake of low-molecular-weight components such as monosaccharides and amino acids present in the supernatant and permeate and in low concentrations in the retentate. Since the reduction of fluid loss was found in the retentate and not in the permeate, which contains higher levels of compounds known to enhance fluid absorption through sodium-solute co-transport, coupled sodium-solute uptake is unlikely to be responsible for the observed protective effect. Secondly, vegetable polysaccharides such as starch (Wapnir et al., 1998; Wingertzahn et al., 1999) and gum arabic (Turvill et al., 2000; Wapnir et al., 1996; Wapnir et al., 1997a) have been shown to enhance intestinal electrolyte and/or water absorption in normal and secreting rat small intestine. Gum arabic has been shown to enhance net sodium absorption without altering net water absorption in normal rat jejunum (Wapnir et al., 1996), whereas water absorption was increased in case of diarrhoea (Turvill et al., 2000; Wapnir et al., 1997a). Similar results were obtained in our previous tempe study, where no differences were seen in net water absorption in uninfected segments for saline and tempe having both the same osmolality, whereas tempe doubled the net water absorption in ETEC-infected segments compared to saline (Chapter 6). Enhanced absorption could be the result of increased accessibility of electrolytes and associated water to the microvillus membrane through the emulsifying properties of gum arabic (Wapnir et al., 1997a), but also other physicochemical properties could play a role (Wapnir et al., 1998). Evidence for the possible involvement of tempe polysaccharides in the protective effect

described in this study could be the presence of high-molecular-weight polysaccharides in the tempe supernatant and in its retentate. Additionally, the HPSEC analysis of pre-digested tempe showed an elution pattern identical to undigested tempe in terms of high-molecular-weight compounds, and the supernatant of undigested tempe showed equal protection against ETEC-induced fluid loss as did the supernatant of pre-digested tempe.

Further research is required to identify the component(s) in the high-molecular-weight fraction isolated from tempe which is responsible for the observed protective effect and to identify the specific mechanism(s) underlying the improved net fluid balance observed. These and previous results (Chapter 6) warrant the protective role that tempe (constituents) could play in ETEC-associated diarrhoea in piglets and possibly in children.

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The authors thank Mirjana Eimermann, Gerrit de Vrij (Numico Research BV), Esther van Andel, Gert-Jan de Graaf, Arie Hoogendoorn and Ank van Zijderveld-van Bemmel (ID-Lelystad) for their excellent technical assistance.

Chapter 8

Effect of fermented soya bean on ETEC-induced diarrhoea and feed efficiency in weaned piglets

K88 positive enterotoxigenic *Escherichia coli* (ETEC K88+) is an important cause of diarrhoea in young piglets especially during the first two weeks after weaning. Tempe, a fungal fermented soya bean food, has been shown to inhibit *E. coli* infection in rabbits, to reduce fluid losses in ETEC K88+ infected piglet small intestinal segments and was reported to be beneficial in terms of shorter duration of diarrhoeal episodes as well as rehabilitation periods in malnourished children and may therefore have beneficial effects in diarrhoea control. In a first phase piglet diet toasted full-fat soya beans (20%) were replaced with either cooked soya beans or *Rhizopus microsporus* fermented soya beans or *Bacillus subtilis* fermented soya beans. The effect on the occurrence and severity of diarrhoea in ETEC K88+ challenged weaned piglets was determined. Severity of diarrhoea was significantly less on the diet with *Rhizopus* fermented soya beans compared with the control diet containing toasted soya beans. Piglets fed fermented soya beans showed increased feed intake (13 and 12%), average daily weight gain (18 and 21%), and feed efficiency (3 and 8%) (for *Rhizopus* and *Bacillus* fermented soya beans, respectively). Cooked and fermented soya beans appeared beneficial in the control of diarrhoea in ETEC K88+ challenged weaned piglets (especially *Rhizopus* fermented) and significantly improved weight gain and feed intake (especially *Bacillus* fermented). These characteristics make them particularly suitable to use in individuals suffering from diarrhoea and malnutrition.

Submitted for publication

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INTRODUCTION

Weaning remains a critical phase in pig production and is associated with digestive disorders causing reduced growth and diarrhoea which is often associated with different *Escherichia coli* bacteria. Postweaning colibacillosis is the most common cause of postweaning mortality on many farms killing 1.5-2.0% of the piglets weaned (Hampson, 1994), causing major economic losses to the pig industry (Van Beers-Schreurs et al., 1992). Enteric diseases due to strains of enterotoxigenic *E. coli* (ETEC) are not only the most common type of colibacillosis in pigs and calves, but also occur widely in man (Nagy and Fekete, 1999) and ETEC is a predominant causative micro-organism of severe diarrhoea in children in developing countries (Bhan, 2000).

Tempe, a fungal fermented soya bean food, has been shown to inhibit *E. coli* infection in rabbits (Karmini et al., 1997; Karyadi et al., 1990), minimise fluid losses in ETEC-infected small intestinal segments of piglets (Chapters 6 and 7) and shorten duration of diarrhoea when supplemented to the diet of malnourished children (Kalavi et al., 1996; Soenarto et al., 1997). Tempe is a traditional fermented food made from soaked and cooked soya beans inoculated with a mould usually of the genus *Rhizopus* (Nout and Rombouts, 1990; Steinkraus, 1996). An important function of the mould in the fermentation process is the synthesis of enzymes, which hydrolyse soya bean constituents and contribute to the development of a desirable texture, flavour, and aroma of the product (Hachmeister and Fung, 1993). Fermentation also may decrease or eliminate anti-nutritional constituents (Hachmeister and Fung, 1993; Mital and Garg, 1990; Reddy and Pierson, 1994). Overall, the nutritional quality of the fermented product is improved. Alkaline fermentations are dominated by *Bacillus* spp. and are characterised by extensive hydrolysis of protein to amino acids and peptides (Steinkraus, 1996). *Bacillus* fermented foods are common in African and Asian countries, however no reports exist on feeding trials involving *Bacillus* fermented foods. Fermentation of soya bean using several *Bacillus* spp. resulted in major biochemical changes in the substrate leading to an increase in solubility and *in vitro* digestibility (Kiers et al., 2000b). Investigations at protein and carbohydrate level revealed significant breakdown of polymers into water-soluble low-molecular-weight peptides and oligosaccharides, as well as amino acids and monosaccharides leading to a product with high nutrient availability in which the need for degradation of nutrients by gastrointestinal enzymes is lowered (Kiers et al., 2000b).

Antibiotics and antimicrobial compounds have been added for a long time to starter pig diets for their health and growth-promoting properties. The continued use of sub-therapeutic levels of antibiotics in animal feeds may contribute to antibiotic resistance in humans (Wierup, 2000). Probiotics have been widely promoted as alternatives to the use of antibiotics or other feed additives (Fuller, 1989) and besides strains of lactic acid bacteria also strains of *Bacillus* spp. appear to be useful (Kyriakis et al., 1999; Zani et al., 1998). By replacing (toasted) soya bean (meal) with fermented soya beans, substantial levels of (potentially probiotic) micro-organisms such as lactic acid bacteria in case of tempe (Mulyowidarso et al., 1990; Nout and Rombouts, 1990) and *Bacillus* spp. in case of *Bacillus* fermented soya beans (Sarkar et al., 1994) as well as highly digestible protein sources are provided (Baumann and Bisping, 1995; Kiers et al., 2000a; Kiers et al., 2000b; Sarkar et al., 1997b; Sparringa and Owens, 1999).

The objective of this study was to determine the effect of diets containing soya beans fermented with *Rhizopus* sp. or *Bacillus* sp. on ETEC-induced diarrhoea in the first two weeks postweaning and on feed utilisation and growth performance during the first four weeks postweaning in piglets.

MATERIALS AND METHODS

Soya bean products

Cooked soya beans

Dehulled full-fat yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater using accelerated acidification at 30°C (Nout et al., 1987). Subsequently, the beans were washed with tapwater and cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), cooled and superficially dried at room temperature.

Rhizopus fermented soya beans (tempe)

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 days at 30°C and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone (PPS). Cooked beans (see *Cooked soya beans*) were transferred to a 450-l size rotary-drum bioreactor (Han et al., 1999) and inoculated with the sporangiospore suspension (1% v/w). Fermentation was carried out at 37°C for 48h. Agitated solid-substrate fermentation did not result in a solid mass as in traditional tempe, but in individually fermented soya beans that could be easily processed into food ingredients.

Table 8.1 Analysis of the soya bean products.

Compound	Toasted	*Rhizopus*	*Bacillus*	Cooked	Method of analysis
		g/100g			
Moisture	11.1	2.9	5.5	2.7	EC 71/393
Crude lipid	20.2	31.6	29.9	30.1	EC 84/4
Crude fibre	4.5	6.6	4.3	2.8	EC 73/46
Ash	4.9	1.9	3.4	2.3	EC 71/250
Crude protein	35.0	46.1	43.8	44.9	EC 72/199
Non-protein nitrogen	0.3	1.2	1.7	0.2	IDF 20 B part 4 [1]
TIA [2], mg/g	2.0	2.5	1.2	1.2	NEN 3573
Amino acids					EEG L257/14-23/1998
Arginine	2.54	2.93	3.05	3.28	
Lysine	2.11	2.59	2.66	2.80	
Methionine + cysteine	0.96	1.07	1.17	1.10	
Glycine	1.46	1.92	1.82	1.96	
Glutamic acid	6.42	7.72	8.05	8.72	

[1] International Dairy Federation, 1993

[2] Trypsin inhibitor activity

Bacillus fermented soya beans

For *Bacillus subtilis* fermentation soya beans were processed as in section *Cooked soya beans* with the difference that soaking was performed without acidification and at 4°C. *Bacillus subtilis* LU B83 (strain DK-W1 kindly provided by Dr. P.K. Sarkar, University of North Bengal, India) was pre-cultured on nutrient agar (Oxoid, CM3) and later cultured in brain heart infusion broth (Difco, 0037-17) for 18h at 37°C. The culture was diluted using PPS and the cooked soya beans were inoculated ($\pm 10^6$-10^7 colony-forming-units (cfu)/g). Fermentation was carried out for 48h at 37°C in large vessels each containing 35 kg of inoculated cooked soya beans.

Table 8.2 Composition of the diets.

	Control	*Rhizopus*	*Bacillus*	Cooked
Ingredient		g/100 g		
Barley	20.0	21.0	21.0	21.0
Wheat	32.0	32.0	32.0	32.0
Maize	10.0	10.0	10.0	10.0
Toasted soya beans	20.0	-	-	-
Rhizopus fermented soya beans	-	16.5	-	-
Bacillus fermented soya beans	-	-	17.5	-
Cooked soya beans	-	-	-	17.0
Water	-	2.5	1.5	2.0
Whey powder	10.0	10.0	10.0	10.0
Fishmeal	6.0	6.0	6.0	6.0
Minerals [1]	1.2	1.2	1.2	1.2
Salt	0.1	0.1	0.1	0.1
Amino acids [2]	0.5	0.5	0.5	0.5
Vitamins [3]	0.2	0.2	0.2	0.2
Calculated analysis		%		
Crude protein	19.0	19.6	19.6	19.6
Crude lipid	7.1	7.3	7.3	7.2
Crude fibre	3.2	3.2	2.9	2.6
Lysine	1.11	1.13	1.13	1.13
Methionine + cysteine	0.60	0.62	0.62	0.62
Ash	5.3	4.5	4.8	4.6
Calcium	0.70	0.69	0.69	0.69
Phosphorus	0.70	0.69	0.70	0.69
Sodium	0.20	0.20	0.20	0.20
Potassium	1.01	0.96	0.98	0.97
Net energy, Kcal/kg	2523	2523	2544	2545

[1] Mineral mix: supplying macrominerals and trace elements up to stated calculated values
[2] Amino acids mix: supplying lysine, methionine and threonine
[3] Vitamin mix: supplying (IU/kg) vitamin A 10000, vitamin D 2500, vitamin E 50 and B vitamins

Subsequent processing of cooked and fermented soya beans
After cooking or fermentation, the products were spread onto perforated trays and were air-dried in a cross flow oven for 24h at 50°C. All three soya bean products as well as commercially obtained dehulled full-fat toasted soya beans that were used as control, were ground using a 1.5 mm screen, analysed (Table 8.1) and subsequently mixed (20%) with the other ingredients and palletised at 60°C. The composition of the diets is shown in Table 8.2.

Model of porcine postweaning colibacillosis

The experimental protocol was approved by the Animal Experimental Committee.

Animals and experimental design
Piglets (Large White X Landrace), obtained form the Swine Research Centre (Nutreco, Boxmeer, The Netherlands), were weaned in the fourth week of life (on average at day 25). Blocks of 4 piglets within litter and sex were assigned and of these blocks piglets were randomly allocated to each of the 4 treatments. Each treatment-group consisted of 24 piglets, so in total 96 piglets were used. The first 2 days after weaning piglets for each treatment were housed in 2 groups of 12 and were orally treated with 60 mg colistine sulphate once daily. During these first 2 days they had free access to the dry feed pellets and water and in addition a small amount of feed pellets soaked in water was offered twice daily. On day 2 after weaning piglets were weighed and housed individually in cages fitted with a drinker and a

feed hopper, and were inoculated with ETEC O149:K91:K88^{ac} (ID-Lelystad). This strain produces heat-labile enterotoxins (LT) as well as heat-stable enterotoxins (STb). Two 5 ml doses of ETEC broth (containing 8.1 $\log_{10}$ (cfu/ml)) were given orally 4h apart. Feed was checked twice daily to ensure *ad libitum* availability. Feed supply per piglet was monitored and piglets were weighed at the end of each week up to 4 weeks after challenge. Lights were on during 16h per day, and the animal house was climatised and mechanically ventilated. Temperature setting gradually decreased from 28 to 24°C during the 4 weeks period.

Clinical and bacteriological examinations

The piglets were clinically examined and signs of sickness were recorded twice daily for each piglet during weeks 1 and 2 and once daily during weeks 3 and 4 after the challenge. Faecal consistency was assessed visually and characterised according to the following scale: 0 = firm and formed faeces, 2 = shapeless faeces, 4 = liquid faeces, 6 = watery diarrhoea. Autopsy was carried out on piglets which died during the experiment. Rectal swabs were taken immediately before and 3, 5, 7 and 10 days after challenging and bacteriological culturing was done on heart infusion agar (Difco, 0044-17-9) supplemented with 5% of defibrinated sheep blood. Plates were incubated for 18-24h at 37°C and haemolytic colonies were recorded and serotyped using specific monoclonal antibodies (ID-Lelystad).

Brush border isolation and adhesion

Four weeks after challenge all piglets were euthanatised by injecting (iv) 10 ml euthenasate, containing 200 mg/ml sodiumpentobarbital. Brush borders were isolated from jejunal epithelial cells and were tested for adhesion *in vitro* (Sellwood et al., 1975). Piglets yielding brush borders that resulted in microscopically detectable adhesion were recorded as K88-receptor positive.

Data processing and statistical analysis

Piglets were considered having **diarrhoea** when the faecal consistency scored > 2. **Diarrhoea incidence** was defined as the percentage of animals with diarrhoea on a specific day. **Faecal score (=diarrhoea severity)** was defined as average faecal consistency score on a specific day. The following three parameters were defined for the 2 week period after challenge: **average diarrhoea incidence, average faecal score (=average diarrhoea severity)** and **days with diarrhoea** which was defined as the average of the number of days with diarrhoea for each piglet. Faecal scores were ranked using the RANK procedure in SAS, and diarrhoea incidence, faecal score (diarrhoea severity) and days with diarrhoea were analysed by one-way analysis of variance using the General Linear Models (GLM) procedure of the SAS system (Version 6.12, SAS Institute Inc., Cary, NC, USA). The level of significance was set at P=0.10.

The parameters bodyweight, daily gain, feed intake and feed efficiency (gain/feed ratio) were analysed using the GLM procedure of the SAS system to calculate Least Square Means and P values for differences between the groups. Variables used were block, sex, weight at weaning and treatment. Differences were considered significant at $P < 0.10$.

RESULTS

Soya bean fermentation

Table 8.1 shows some characteristics of the differently processed and fermented soya bean products. Crude lipid and crude protein were higher in cooked and fermented soya beans compared to the toasted beans. No differences were observed in crude lipid and crude protein between cooked and fermented soya beans, but values for crude fibre were higher for fermented soya beans. Although crude protein as well as total amino acids contents were similar between cooked and fermented soya beans, fermentation led to a major increase in non-protein nitrogen.

Table 8.3 Number of piglets showing *E. coli* K88 in rectal swabs (percentage given in brackets). Number of piglets is shown in italic.

	Days after challenge				
	0	3	5	7	10
Toasted	0 (0)	13 (54)	11 (48)	2 (9)	0 (0)
	24	*24*	*23*	*23*	*23*
Rhizopus	1 (4)	7 (29)	6 (26)	0 (0)	0 (0)
	24	*24*	*23*	*23*	*22*
Bacillus	0 (0)	12 (50)	9 (38)	1 (5)	0 (0)
	24	*24*	*24*	*21*	*20*
Cooked	0 (0)	8 (33)	8 (33)	1 (4)	0 (0)
	24	*24*	*24*	*24*	*23*
Total	1 (1)	40 (42)	34 (36)	4 (4)	0 (0)
	96	*96*	*94*	*91*	*88*

Diarrhoea

Before challenging the piglets, K88+ *E. coli* was detected in the rectal swab of one piglet only (Table 8.3). Three days after challenge 42% of all piglets showed faecal excretion of K88+ *E. coli* but 10 days after challenge K88+ *E. coli* was not detected anymore. During the first week after challenge, detection of K88+ *E. coli* was lowest in piglets fed the *Rhizopus* fermented soya beans.

Unfortunately, only of 60 piglets the receptor status could clearly be determined. Diarrhoea parameters of receptor-positive and receptor-negative piglets irrespective of the consumed diets are shown in Table 8.4. All parameters were markedly lower for the receptor-negative piglets. The receptor-positive piglets were more or less homogeneously distributed among the four groups consuming the different types of soya bean (Table 8.5).

Table 8.4 Average diarrhoea incidence, average faecal score and days with diarrhoea for receptor-positive and receptor-negative piglets.

	Receptor-positive	Receptor-negative
N	26	34
Diarrhoea parameters [1]		
Average diarrhoea incidence (%)	41±22	32±15
Average faecal score	2.1±1.1	1.6±0.7
Days with diarrhoea (days)	5.8±3.2	4.4±3.2

[1] Average ± standard deviation

Diarrhoea incidence and faecal score are shown in Figure 8.1. All three experimental diets showed reduced incidence of diarrhoea three to five days after challenge. The average diarrhoea incidence was reduced for all three experimental groups, but the differences were not statistically significant (Table 8.5). Average faecal score was significantly less in the *Rhizopus* group compared to the toasted group (P=0.06) but no significant differences were observed between the three experimental groups. The piglets in the three experimental groups experienced less diarrhoea days and, although not statistically significant, this was almost two days shorter for the *Rhizopus* group (4.3±3.5 days) compared to the toasted group (6.2±3.1 days) (Table 8.5).

Table 8.5 Distribution of deaths, receptor-positive and receptor-negative piglets, as well as the average diarrhoea incidence, average faecal score and days with diarrhoea for the groups of piglets fed the different diets.

	Toasted	*Rhizopus*	*Bacillus*	Cooked
N	24	24	24	24
Deaths	1	3 [1]	4	1
K88-receptor positive	6	7	8	5
K88-receptor negative	11	6	6	11
Diarrhoea parameters [2,3]				
Average diarrhoea incidence (%)	46±22	33±12	38±16	37±16
Average faecal score	2.3±1.1[a]	1.7±0.6[b]	1.8±0.8[ab]	1.9±0.8[ab]
Days with diarrhoea (days)	6.2±3.1	4.3±3.5	4.8±2.7	5.0±3.0

[1] One piglet in the *Rhizopus* group died 13 days after challenge from pleuropneumonia
[2] Average ± standard deviation
[3] Values with different superscripts differ significantly

Mortality

Mortality up to the end of the trial in total amounted to 9.4% (9/96) (Table 8.5). Eight of these deaths occurred from 3-8 days post-challenge after a couple of days with faecal score of 4-6 and clinical signs indicated coli-bacillosis. On two piglets no autopsy was done because they died during the weekend, and in the 6 autopsied cases coli-enteritis was diagnosed. The ninth piglet died 2 weeks after the challenge because of pleuropneumonia and belonged to the *Rhizopus* fermented group.

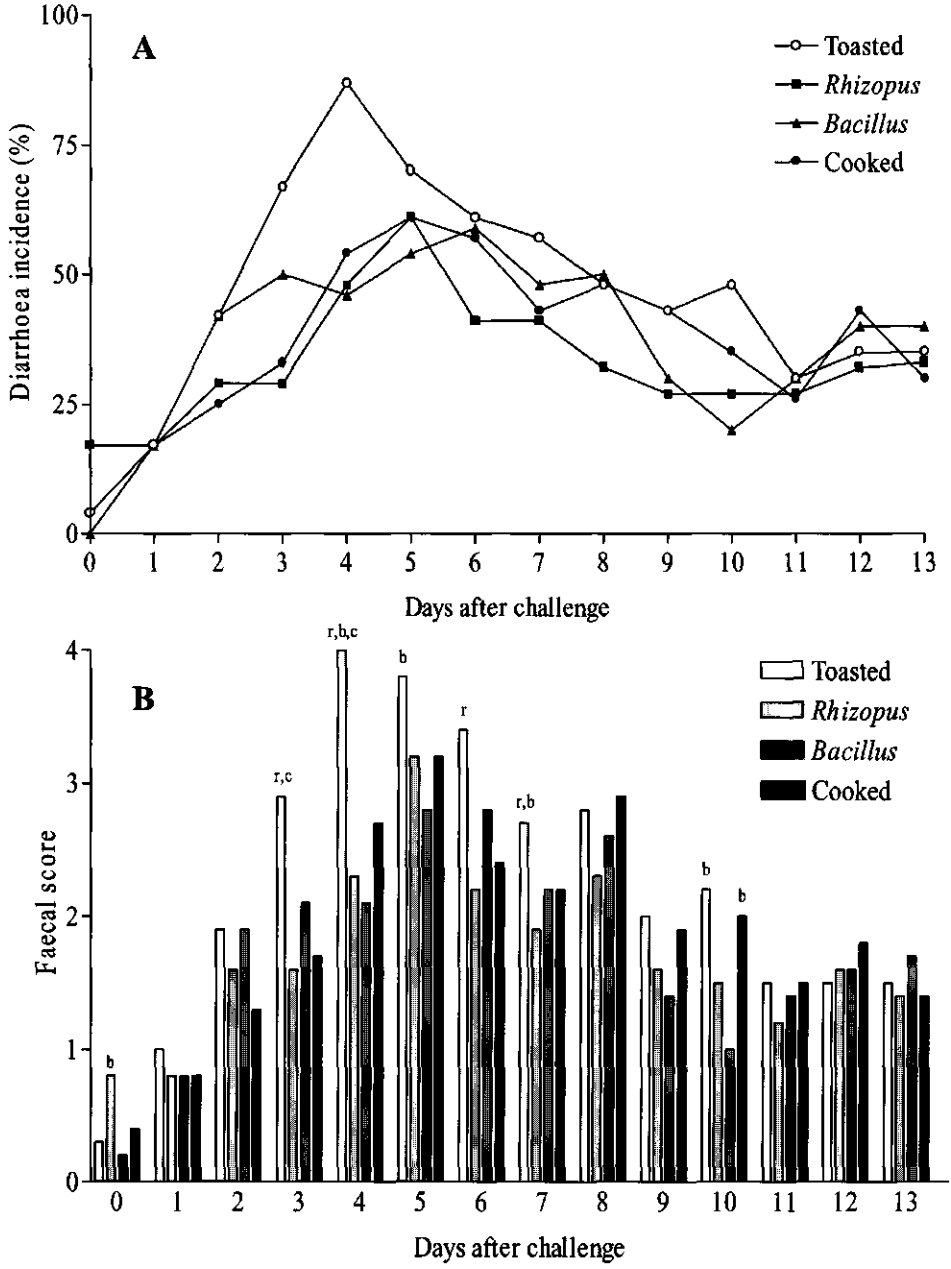

Figure 8.1 Diarrhoea incidence (A) and faecal score (B) of piglets fed toasted, *Rhizopus* fermented, *Bacillus* fermented or cooked soya beans during the first 2 weeks after challenge (day 0). Significant differences ($P<0.10$) are indicated in figure B (r: different from *Rhizopus*, b: different from *Bacillus*, c: different from cooked).

Table 8.6 *Growth performance data (least square means ± standard error of the means). Values in one row with different superscripts differ significantly (P<0.10)*

	Toasted	*Rhizopus*	*Bacillus*	Cooked
Mean bodyweight (kg)				
Day 0 (challenge)	8.07±0.15	8.14±0.16	8.07±0.16	7.95±0.15
Day 7	8.73±0.10^{a}	8.75±0.11^{a}	9.01±0.11^{b}	9.01±0.10^{b}
Day 14	10.37±0.19^{a}	10.64±0.21^{ab}	10.91±0.21^{b}	10.89±0.20^{b}
Day 21	13.66±0.29^{a}	14.30±0.32^{ab}	14.57±0.32^{b}	14.14±0.30^{ab}
Day 28	17.53±0.38^{a}	19.17±0.42^{b}	19.47±0.42^{b}	18.70±0.39^{b}
Average daily gain (g/day)				
Days 0-7	81±14^{a}	83±16^{a}	121±16^{b}	121±15^{b}
Days 7-14	235±20	270±22	272±22	268±20
Days 14-21	469±23^{ab}	522±25^{a}	522±25^{a}	464±23^{b}
Days 21-28	553±24^{a}	696±26^{b}	700±27^{b}	652±25^{b}
Days 0-28	334±14^{a}	393±15^{b}	404±15^{b}	376±14^{b}
Average daily feed intake (g)				
Days 0-7	167±10	166±11	188±11	181±10
Days 7-14	307±17	347±18	336±18	342±17
Days 14-21	564±26^{a}	639±28^{b}	632±29^{b}	598±27^{ab}
Days 21-28	814±29^{a}	941±32^{b}	910±32^{b}	882±30^{b}
Days 0-28	463±17^{a}	523±19^{b}	517±19^{b}	501±18^{ab}
Gain/feed ratio				
Days 0-7	0.13±0.13^{a}	0.17±0.14^{a}	0.60±0.14^{b}	0.54±0.13^{b}
Days 7-14	0.72±0.05	0.71±0.05	0.78±0.05	0.77±0.05
Days 14-21	0.85±0.02^{a}	0.84±0.03^{ab}	0.83±0.03^{ab}	0.79±0.02^{b}
Days 21-28	0.68±0.02^{a}	0.74±0.02^{b}	0.77±0.02^{b}	0.74±0.01^{b}
Days 0-28	0.72±0.01^{a}	0.74±0.01^{b}	0.78±0.01^{c}	0.75±0.01^{b}

Growth performance (Table 8.6)

Mean bodyweight at the end of the experiment was significantly higher for the experimental groups compared to the control group. Although mean bodyweight was higher for piglets fed fermented soya beans compared to cooked soya beans, this difference was not statistically significant. Average daily gain and average daily feed intake were low for piglets fed toasted and *Rhizopus* fermented soya bean during the first week, which resulted in low feed efficiency for the first week. However, no significant differences in these parameters were found between all four groups in the second week. During the third and fourth week significant differences appeared in average daily gain, average daily feed intake and consequently in feed efficiency, with significantly higher values for all these parameters in the three experimental groups. Over the total 4-week period piglets fed fermented soya beans showed increased average daily feed intake (13 and 12%), average daily weight gain (18 and 21%), and feed efficiency (3 and 8%) (for *Rhizopus* and *Bacillus* fermented soya beans, respectively) when compared to piglets fed toasted soya beans.

DISCUSSION

The etiology of postweaning diarrhoea is complex and multifactorial (Hampson, 1994; Nabuurs et al., 1993c; Pluske et al., 1997; Van Beers-Schreurs et al., 1992). In particular, the contribution of ETEC is difficult to assess and models have been described to simulate the most common patterns of the disorders by inoculating weaning piglets with pathogenic strains of *E. coli* (Madec et al., 2000). The experimental model used in our study is based on former research (Meijer et al., 1997). It was considered useful to study effects of experimental feed

(supplements) on incidence, duration and severity of diarrhoea during the first 2 week period postweaning and has previously been shown to induce diarrhoea in 80% of the piglets infected (JCM personal communications). The duration of the experiment was extended for another 2 weeks to evaluate the effect of fermented soya beans on feed utilisation and growth performance as well.

Three days after the ETEC challenge, 42% of all piglets excreted *E. coli* K88+ and 10 days after inoculation no K88+ bacteria were detected anymore reflecting the transient nature of the infection. The percentage of piglets excreting *E. coli* K88+ in the groups fed cooked soya beans or tempe was lower compared to piglets fed toasted soya beans. It could be that certain factors, for instance lactic acid bacteria present in cooked and in higher numbers in *Rhizopus* fermented soya beans, showed antagonistic properties against ETEC. Probiotic preparations including *Bacillus* spp. have been suggested to inhibit pathogens in the gut (Fuller, 1989; Katelaris, 1996; Kyriakis et al., 1999; Tannock, 1997; Wood, 1992; Zani et al., 1998). However, in our study similar number of piglets excreted *E. coli* K88+ in the group fed *Bacillus* fermented soya beans compared to the group fed toasted soya beans.

As was shown before (Madec et al., 2000; Sarmiento et al., 1988) diarrhoea was more prevalent in piglets expressing receptors involved in the adhesion of ETEC. Unfortunately we were unable to get a clear picture on the distribution of the receptor-positive and receptor-negative piglets among the groups. Although the piglets identified as receptor-positive were quite homogeneously distributed among the groups, the receptor status of a large number of piglets could not be determined, including the piglets which died during the trial. The piglets which died of coli-bacillosis were most probably receptor-positive as well. The higher number of deaths in the groups fed fermented soya beans compared to the groups fed heat-treated soya beans should be taken into account.

Not only ETEC excretion was reduced in the three experimental groups, also the average diarrhoea incidence, average faecal score and the average number of days with diarrhoea were lower, which could reflect possible antagonism towards ETEC or could be the result of beneficial effects of cooked and especially fermented soya beans on fluid balance (reduced secretion or increased absorption), nutritional status or immune system. Also studies in malnourished children revealed beneficial effects of tempe on duration of diarrhoeal episodes and rehabilitation period (Kalavi et al., 1996; Karyadi and Lukito, 1996; Soenarto et al., 1997).

The present study showed increased average daily weight gain and improved feed utilisation (gain/feed ratio) in piglets fed cooked soya beans compared to piglets fed toasted soya beans. This could be the result of increased breakdown of anti-nutritional factors (although trypsin inhibitor level for all soya bean products was sufficiently low) and/or an increased digestibility due to the more extensive and intense heat treatment during cooking compared to toasting. Leaching of water-soluble non-digestible matter during cooking may also contribute to improved digestibility of cooked soya beans. Small improvements of *in vitro* digestibility have been reported for tempe and *Bacillus* fermented soya beans compared to their unfermented controls (Kiers et al., 2000a; Kiers et al., 2000b). *In vivo* trials using rats and neonatal pigs mostly showed slight improvements in growth, daily weight gain and protein efficiency when fungal fermented soya beans and cowpeas were fed compared to unfermented controls (Murata et al., 1971; Nout and Rombouts, 1990; Smith et al., 1964; Steinkraus, 1996; Zamora and Veum, 1979; Zamora and Veum, 1988). The increased bodyweight at the end of the trial (3-4%) and the increased average daily weight gain (5-8%) of piglets fed fermented soya bean compared to cooked soya bean, might reflect the improved nutrient availability, as was shown *in vitro* for tempe and *Bacillus* fermented soya beans (Kiers et al., 2000a; Kiers et al., 2000b), as a result of the action of micro-organisms during

fermentation leading to a 'pre-digestion' of the substrate (De Reu, 1995; Hachmeister and Fung, 1993; Kiers et al., 2000a; Kiers et al., 2000b; Mital and Garg, 1990; Nout and Rombouts, 1990; Smith et al., 1964). The significantly improved feed utilisation (8% compared to toasted soya bean) of *Bacillus subtilis* fermented soya beans was very likely due to the extensive hydrolysis of protein resulting in readily available free amino acids and peptides which is a major characteristic of these fermentations (Kiers et al., 2000b; Sarkar et al., 1997b; Steinkraus, 1996). *Bacillus* strains have been used and proven effective to improve weight gain and feed conversion (Kyriakis et al., 1999; Zani et al., 1998).

In conclusion, cooked and (especially *Rhizopus*) fermented soya beans appeared beneficial in the control of ETEC-induced diarrhoea and cooked and (especially *Bacillus*) fermented soya beans significantly improved weight gain and feed intake. These characteristics make fermented soya beans particularly suitable to be used in weaning piglets suffering from diarrhoea and malnutrition and in children in developing countries where many children die because their bodies are undernourished and weakened due to diarrhoeal diseases.

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Chapter 9

General discussion

Particularly during the last decade an increased interest in nutritional and health effects of food and feed has evolved. An important question is what fermentation has to offer in this field. A variety of indigenous fermented foods exist, many of which date back hundreds or even thousands of years. An important function of the micro-organisms involved in the fermentation process is the synthesis of enzymes that hydrolyse the food ingredients and contribute to the development of a desirable texture, flavour, and aroma of the product. Fermentation also decreases anti-nutritional constituents, and the nutritional quality and digestibility of the fermented products is improved. Fermented foods could enhance the barrier function of the gastrointestinal tract, possibly resulting in reduced diarrhoeal disease risks.

Fermented soya bean foods are attractive products in view of sensory quality, nutritional properties and anti-diarrhoea characteristics. Furthermore, the present or potential role of fermented soya bean foods in prevention and treatment of chronic diseases gains increasing attention.

BIOCHEMICAL CHANGES DURING SOYA BEAN FERMENTATION

During the fungal fermentation process a substantial breakdown occurs of the soya bean storage proteins, lipids, and carbohydrates due to the production of a variety of proteases, lipases and carbohydrases (De Reu, 1995). Total protein content of unfermented soya beans and tempe is more or less the same but a 30-35 fold increase in free amino acids occurs during fermentation. However, this increase in free amino acids is far less than the 50-60 fold increase observed during *Bacillus* fermentation (Figure 9.1). *Bacillus* spp. show considerably higher proteolytic activity compared to the *Rhizopus* spp. used, and virtually all major protein subunits are degraded (Chapter 3). During fermentation with *Rhizopus microsporus* a remarkable increase in alanine was observed whereas *Bacillus subtilis* fermentation led to major increases in almost all amino acids analysed and especially in glutamic acid as was also shown before (Sarkar et al., 1997b). Increased release of amino acids could improve the nutritional value of fermented soya beans.

In soya beans high levels of α-galactosides of sucrose (raffinose, stachyose) are found. These may have prebiotic properties, but they also contribute to intestinal gas production (flatulence). Anyway, these oligosaccharides are mainly removed by soaking and cooking of soya beans (Ruiz-Teran and Owens, 1999; Sarkar et al., 1997a) and are probably further degraded by the moulds used in tempe manufacture (Rehms and Barz, 1995; Ruiz-Teran and Owens, 1999) and certainly by *Bacillus subtilis* fermentation in the production of kinema (Sarkar et al., 1997a).

In contrast to numerous reports on (flatulence-causing) oligosaccharides, hardly any attention has been paid to polysaccharide degradation during soya bean fermentation. During *Bacillus* fermentation considerable degradation of insoluble soya bean polysaccharides occurs resulting in the accumulation of water-soluble low-molecular-weight carbohydrates (Figure 3.3). During tempe fermentation a completely different pattern of degradation products is generated, with a large range of water-soluble high-molecular-weight polysaccharides being liberated (Figure 7.3). Major carbohydrases of *R. oligosporus* in tempe include polygalacturonase, endocellulase, xylanase and arabinase (Nout and Rombouts, 1990; Sarrette et al., 1992), and during enzymatic maceration predominantly the arabinogalactan and pectin fractions of the soya bean are solubilised (De Reu et al., 1997).

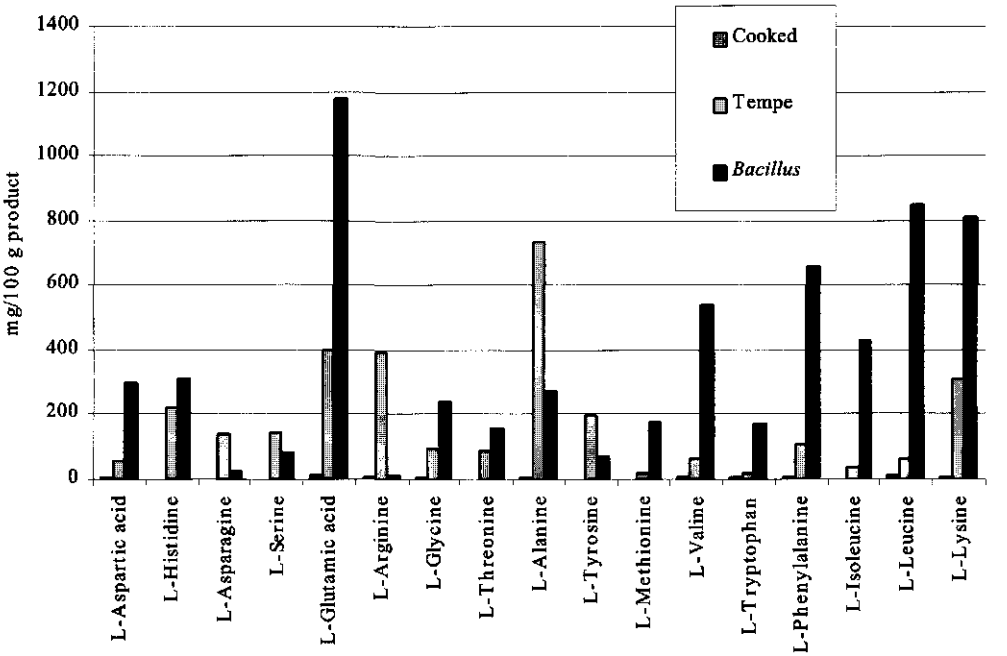

Figure 9.1 Free amino acids profile of cooked soya beans, tempe and *Bacillus* fermented soya beans.

Both types of fermentation increase levels of water-soluble nitrogen, low-molecular-weight peptides, free amino acids, soluble carbohydrates and low-molecular-weight saccharides. However, distinct differences are observed between *Rhizopus* fermentation and *Bacillus* fermentation probably as a result of the expression of different types and levels of enzymes resulting in two types of fermented foods with unique biochemical and possibly nutritional properties.

DIGESTION AND ABSORPTION

The digestibility model developed and described in Chapter 2 has been used as a technological tool to describe effects of food processing operations on solubility and digestibility in a comparative way. Results should be interpreted with caution because of limited possibility to extrapolate *in vitro* data to the physiological *in vivo* situation. In the *in vitro* digestion model we used rather mild conditions concerning pH and enzymatic incubation periods. This was done to mimic the conditions in the un(der)developed gastrointestinal tract of children and young animals. In Table 9.1 *in vitro* parameters are compared to the *in vivo* situation with respect to the stomach contents and duodenal chyme in 6-week-old piglets a few hours after feeding a standard piglet diet. It can be seen that the characteristics of the *in vitro* pre-digested soya bean products fall within the physiological range observed *in vivo* except for osmolality which is rather low *in vitro*.

In vitro digestions of tempe (Chapter 2) and *Bacillus* fermented soya bean (Chapter 3) indicate small increases of *in vitro* dry matter digestibility, whereas dry matter solubility and absorbability are greatly enhanced as a result of protein and carbohydrate degradation during

fermentation as discussed above. As was discussed in Chapters 2 and 3, fermentation was nearly capable to reach nutrient availability to the level obtained after *in vitro* digestion of cooked soya beans. No other data are available on *in vitro* digestion of fermented legume foods except for reports describing improved *in vitro* digestibility of starch and proteins during lactic acid fermentation of wadies, popular indigenous fermented legume products from India (Sunita and Neelam, 1995; Yadav and Khetarpaul, 1994). Tempe showed higher dry matter uptake compared to cooked soya bean during perfusion of piglet small intestinal segments (Chapter 6) probably reflecting increased nutrient availability. In fact, beneficial effects of fermented soya beans were observed on growth and feed efficiency in early weaned piglets *in vivo* indicating a possible improved nutrient availability/digestibility (Chapter 8).

Table 9.1 *In vitro* and *in vivo* pH, dry matter percentage and osmolality in the stomach and duodenum. *In vitro* values were obtained after *in vitro* digestion of processed and fermented soya beans (Chapter 5) and *in vivo* values are derived from 6-week-old piglets fed a standard piglet diet (n=26, average ± standard deviation, range is shown between brackets).

	Stomach			Duodenum		
	pH	Dry matter content (%)	Osmolality (mOsmol/kg)	pH	Dry matter content (%)	Osmolality (mOsmol/kg)
In vitro	4.0-4.2	± 11	ND [1]	6.1 - 6.3	6.4 - 6.9	152-294
In vivo	3.2±0.7	15.9±5.4	274±55	6.4±0.6	8.7±4.4	450±93
	(2.2-5.4)	(4.4-26.0)	(139-422)	(5.1-7.5)	(2.5-12.7)	(307-636)

[1] Not determined

ETEC ASSOCIATED DIARRHOEA

Enterotoxigenic *Escherichia coli* (ETEC) frequently occurs in diarrhoeal disease afflicting young children (Bhan, 2000; Huilan et al., 1991), travellers (Gaastra and Svennerholm, 1996) and domestic animals (Hampson, 1994; Nabuurs et al., 1993c; Nagy and Fekete, 1999). The two important determinants of virulence that play a role in the development of the infection are the ability to colonise the small intestine mediated by specific fimbrial adhesins and the production of enterotoxins. These toxins stimulate the active secretion of chloride by the crypt cells and inhibit the normal absorption of chloride coupled to sodium by villous cells. This results in the net secretion of sodium, chloride and water, eventually leading to diarrhoea and dehydration when (re-)absorption in the large intestine is insufficient.

A high protective effect of cooked and fermented soya beans on net fluid loss induced by ETEC was observed (Chapter 6). Furthermore, tempe showed highest protection against diarrhoea in ETEC challenged piglets *in vivo*, whereas cooked soya bean was effective as well (Chapter 8). Besides these beneficial effects on fluid balance of both cooked soya bean and tempe, tempe resulted in reduced electrolyte losses, high uptake of solutes and dry matter during perfusion of piglet small intestinal segments (Chapter 6) and improved average daily weight gain (Chapter 8). Because of these additional benefits of tempe, the protective effect of tempe on fluid loss was studied in some more detail. It was shown that in addition to insoluble compounds also a fraction containing high-molecular-weight solubles was responsible for the protective effect observed for tempe (Chapter 7). These compounds were not released upon pre-digestion of tempe, but were liberated during the fungal fermentation since they were not present in extracts from cooked soya beans. Therefore, the protective effects of cooked soya bean on net fluid loss (Chapter 6) and diarrhoea (Chapter 8) probably will have a different origin. Observed anti-diarrhoeal activity of tempe could be attributable to an anti-secretory or a pro-absorptive effect (or both) on the intestine. Several possibilities for the mechanism of action of the beneficial effects of tempe on ETEC infection are outlined.

Possible modes of action leading to reduced secretion

Antimicrobial properties

In this thesis some attention has been paid to the possible antimicrobial effects attributed to tempe, which might play a role in the development of diarrhoeal diseases as was suggested in several studies (Kalavi et al., 1996; Karmini et al., 1997; Karyadi and Lukito, 1996; Karyadi et al., 1990; Soenarto et al., 1997). However, it appeared that only the growth of some Gram-positive species was affected and no effects on the growth of *E. coli* were observed (Chapter 4; Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969). Possible interference with ETEC pathogenesis due to inhibition of growth does not seem very likely, which was supported by the detection of high numbers of *E. coli* after perfusion of small intestinal segments with pre-digested tempe products (Chapters 6). A potential beneficial role of tempe in diarrhoea prevention or treatment due to antimicrobial characteristics therefore seems very unlikely.

Adhesion intervention

Adherence by ETEC is a prerequisite for its colonisation of mucosal surfaces. The ability of ETEC to adhere to the intestinal epithelium of the host is an important virulence determinant, and adhesion is mediated by proteinaceous surface appendages called fimbriae. The K88 fimbrial adhesin is a major virulence factor of certain strains of ETEC that infect and colonise neonatal pigs. An extract of tempe (fermented with *Rhizopus microsporus* LU 573) was able to strongly inhibit ETEC K88+ haemagglutination and also inhibited ETEC K88+ adherence to brush borders (Chapter 4). Depending on the mould strain used, fungal fermentation of soya beans could therefore result in the liberation or solubilisation of substances which interfere with ETEC adhesion. This could be realised in three ways. First, the substances formed could have an antibody-like activity which might explain the aggregation of ETEC cells observed in several cases. Second, the active substances could act as receptor analogues and could therefore interfere with the fimbrial adhesins preventing adhesion to the mucosal receptors. Finally, interference with epithelial cell surface receptors (adhesin analogues) could have prevented the adhesion as well. Furthermore, proteases such as bromelain have been shown to degrade intestinal receptors in the gut (Chandler and Mynott, 1998; Mynott et al., 1997; Mynott et al., 1996), and since α-galactosidase and protease activity was found in tempe (extracts) (Chapter 7) this might explain the observed protective effects. Inhibiting the attachment of ETEC to the intestinal mucosa by modifying the receptor attachment sites has been the key for developing novel approaches to prevent ETEC-induced diarrhoea (Jin and Zhao, 2000).

Despite the promising results described in Chapter 4, high numbers of ETEC were found after sampling the mucosa of the intestinal segments at the end of the SISP experiments (Chapter 6). In addition, the high-molecular-weight fraction of pre-digested tempe showed protection against fluid loss in contrast to the low-molecular-weight fraction, while both fractions showed 50% reduction in ETEC adhesion to isolated brush borders *in vitro* (Chapter 7). This 50% reduction is about the same as found for undigested cooked soya bean in Chapter 4. The factors in undigested tempe responsible for high inhibition of adhesion (Chapter 4) could either have been degraded during the pre-digestion step, or their concentration was too low to be effective (e.g. due to the dilution during pre-digestion).

Toxin interaction

Described beneficial effects of tempe in relation to protection against ETEC-induced fluid losses could be the result of tempe compounds acting as toxin receptor analogues or as toxin

inactivators (Heerze et al., 1994; Takeda et al., 1999). Further research could be aimed at identifying a possible effect of tempe constituents on toxins.

Interference with intracellular events
The mechanisms which enable enterotoxins to trigger intestinal secretion of water and electrolytes are only partially understood (Peterson and Whipp, 1995). Heat labile toxin (LT) evokes the synthesis of cyclic AMP (cAMP) which leads to activation of protein kinase A resulting in phosphorylation of chloride channels. This results in increased chloride secretion by the crypt cells and inhibition of neutral sodium chloride absorption by the villous cells, eventually leading to diarrhoea through passive (paracellular) secretion of water. Heat stable toxin STa is known to elevate production of cyclic GMP leading to the secretion of water and electrolytes. The mechanism of action of STb is much less known (Nagy and Fekete, 1999). Tempe (constituents) could have a specific inhibitory effect on one of the intracellular events evoked by one or both of the enterotoxins produced by ETEC. For a factor purified from boiled rice such a mechanism has been demonstrated and possibly this factor blocked the activation of chloride channels from the luminal surface of the cell (Mathews et al., 1999).

Probiotic properties
Considerable numbers of lactic acid bacteria and bacilli are present in respectively tempe and *Bacillus* fermented soya bean foods. It could be that some of these bacteria exert probiotic effects such as competition for receptor sites (Blomberg et al., 1993; Fujiwara et al., 1997) and nutrients or by positively influencing the immune system (Yasui et al., 1999). Oral bacteriotherapy using probiotics were used successfully in acute diarrhoea in humans and in pigs (Buydens and Debeuckelaere, 1996; Goldin, 1998; Kyriakis et al., 1999; Rautanen et al., 1998; Shornikova et al., 1997; Zani et al., 1998). It could be questioned whether effects of this kind require living bacteria or that dead bacteria or bacterial (cell wall) fragments are sufficient. All studies described in this thesis were carried out with 'raw' fermented foods, meaning that they have not been fried or cooked which is normally done prior to consumption.

Possible modes of action leading to enhanced absorption

Osmolality
Reduced osmolarity oral rehydration solutions (ORSs) are clinically more effective than WHO-ORS (Sarker et al., 2001; Valentiner-Branth et al., 1999). Furthermore, enhanced clinical efficacy of complex carbohydrate ORSs is most probably the result of their hypotonicity (Thillainayagam et al., 1998). A controlled clinical trial is the only way to determine whether a new ORS is superior to an established solution, but attempts have been made to pre-screen new ORS in a variety of animal models, most of which involve intestinal perfusion (Farthing, 1990). Overall, these animal models have emphasised the potential benefit of using a hypotonic ORS (Farthing, 1990; Pillai et al., 1994a; Thillainayagam et al., 1993). Also, human perfusion studies (Hunt et al., 1992; Hunt et al., 1994) in secreting intestine showed clear benefits of using ORSs with reduced osmolality. The inverse relationship between osmolality and net fluid absorption in the small intestine was demonstrated and confirmed in piglets in this thesis (Chapter 5). Fermentation resulted in increased osmolality and therefore net fluid absorption as such was lower during tempe perfusion compared to cooked soya beans (Chapter 6). The low osmolality of cooked soya beans would therefore be beneficial with respect to fluid absorption (both in normal and in infected small intestine), but this characteristic cannot explain the reduced fluid loss induced by ETEC (Chapters 5 and 6).

Substrate-linked sodium absorption
In enterotoxin-mediated secretory diarrhoea, the substrate-linked sodium absorptive mechanisms on the villous epithelium (Figure 1.5) are left intact. This principle provides the rationale for the use of glucose based ORS to rehydrate the individual. The presence of high levels of peptides, amino acids, disaccharides and monosacharides in fermented soya bean could enhance water absorption is this way. Alanine has been shown to stimulate water and sodium absorption in a model of secretory diarrhoea (Wapnir et al., 1990), and was liberated to a large extent by fermenting soya bean with *Rhizopus* sp. (Figure 9.1). Although this feature could have played a role, it was shown that the high-molecular-weight fraction in tempe was most important. Furthermore, amino-acid-containing ORS formulations are not recommended, since they have no clinical advantage over WHO-ORS for children with acute non-cholera diarrhoea and are also more costly (Bhan et al., 1994).

Vegetable polysaccharides
Starch (Wapnir et al., 1998; Wingertzahn et al., 1999) and gum arabic (Turvill et al., 2000; Wapnir et al., 1996; Wapnir et al., 1997a) have been shown to enhance intestinal electrolyte and/or water absorption in normal and secreting rat small intestine. Enhanced absorption could be the result of increased accessibility of electrolytes and associated water to the brush border membrane through the emulsifying properties of gum arabic (Wapnir et al., 1997a), but also other physicochemical properties could play a role (Wapnir et al., 1998). Soluble high-molecular-weight polysaccharides present in tempe could therefore be responsible for the observed beneficial effect on fluid balance, although the mode of action remains unclear.

INTEGRITY OF THE SMALL INTESTINAL MUCOSA

Marked changes in the histology and biochemistry of the small intestine, such as villous atrophy and crypt hyperplasia, are associated with weaning (Hampson, 1986; Nabuurs et al., 1993a). These changes decrease digestive and absorptive capacity and contribute to postweaning diarrhoea (Hampson, 1986; Pluske et al., 1997). Besides the rather direct effects of tempe on ETEC associated diarrhoea described in Chapters 4, 6 and 7 (inhibition of growth and adhesion, influencing fluid secretion/absorption), tempe could have a more basal effect on the recovery of the small intestinal mucosal barrier function after weaning as well (Bosi, 2000). Tempe, compared to cooked soya beans, contains high levels of easily accessible compounds such as peptides and free amino acids and possibly other factors which could affect intestinal growth and cell proliferation, and was therefore studied in a small feeding trial.

In piglets fed either toasted, cooked or fermented (*Rhizopus microsporus* LU 573) soya beans, villous height was significantly lower on day 4 and 7 after weaning compared to the day of weaning (Table 9.2). The obtained data do not indicate any beneficial effects of tempe on maintaining or quickly restoring villous height in piglets after weaning.

Bacillus fermentation of soya beans resulted in large increases of free glutamine (Figure 9.1). Glutamine has been described as the principal energy source for small intestinal enterocytes and is considered essential for gut metabolism, structure and function (Evans and Shronts, 1992). It has been reported to prevent jejunal atrophy in weaned pigs (Wu et al., 1996), and to reduce rapidly the protein kinase C mediated hyperpermeability in intestinal cells (Kouznetsova et al., 1999; Yang et al., 1999). Possibly, *Bacillus* fermented soya beans have more potential than tempe in maintaining small intestinal epithelial structure and

function. Unfortunately a group of piglets fed *Bacillus* fermented soya beans could not be included in the small feeding trial described above.

Table 9.2 Average villous height, crypt depth and villous/crypt ratio at five sites (10, 25, 50, 75 and 95%) along the entire length of the small intestine of piglets at 4 and 7 days after weaning fed toasted, cooked and fermented (tempe) soya bean. (Average ± standard deviation, n=4) [1]

Day	Diet	Villous height (μm)	Crypt depth (μm)	Villous/crypt ratio
0	-	706±96	92±5	7.7±0.5
4	Toasted	424±35	100±4	4.2±0.6
	Cooked	424±7	100±5	4.2±0.5
	Tempe	396±47	98±5	4.0±0.3
7	Toasted	538±52	103±7 [a]	5.2±0.4 [a]
	Cooked	496±62	95±6 [b]	5.2±0.4 [a]
	Tempe	486±75	102±7 [a]	4.8±0.5 [b]

[1] Different superscripts represent significant differences between the groups

CONCLUDING REMARKS

Fermentation of soya beans, either with *Rhizopus* sp. or with *Bacillus* sp., leads to major degradation of polymeric macronutrients resulting in increased nutrient availability up to the level obtained after *in vitro* digestion of cooked soya beans. Tempe showed higher absorption of nutrients during intestinal perfusion as well, and showed increased (average daily) weight gain when fed to early weaned piglets. *Bacillus* fermented soya beans showed even higher values with respect to growth and feed conversion. Fermentation could be used in producing highly digestible soya bean foods with a potential in feeding individuals suffering from decreased luminal and/or brush border digestion, for instance young animals and children with un(der)developed digestive systems or villous atrophy (e.g. in case of weaning and/or rotavirus infection).

ETEC is a major cause of acute secretory diarrhoea in early weaned piglets and children in developing countries, and was the main target to which tempe was tested. No antimicrobial effects of tempe extracts were observed, but *in vitro* several extracts of tempe were shown to strongly inhibit the adhesion of ETEC, which is thought to be an essential step in pathogenesis. Osmolality was inversely correlated to intestinal fluid absorption. Foods and beverages of low osmolality enhance fluid absorption and therefore could reduce fluid loss in case of acute diarrhoea. ETEC markedly reduced net fluid absorption, but this was not the case in intestinal segments perfused with either pre-digested cooked soya beans or tempe. The protective effect of tempe appeared to be derived from the insoluble matrix and soluble high-molecular-weight compound(s). The mechanism(s) to explain this observed protective effect remains unknown. Several possibilities have been outlined and discussed, and further research is needed. Tempe reduced the incidence and the severity (significantly) of ETEC induced diarrhoea in early weaned piglets compared to toasted soya beans.

Fermented soya bean foods have a potential in feeding early weaned piglets and children in developing countries, that highly need food products that are nutritious, that can be easily digested and absorbed, and that offer protection against diarrhoea.

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Summary

The origins of fermented foods go back to prehistoric times, with early evidence of the alcoholic fermentations of barley to beer and grapes to wine. For hundreds or even thousands of years Oriental people have consumed soya beans in various forms of traditional fermented foods. Fermented soya bean foods include among others tempe and kinema, and these two types were studied with respect to their effects on gastrointestinal digestion and absorption and their potential benefits in case of diarrhoea.

An *in vitro* method was set up to carry out comparative determinations of the dry matter digestibility of cereal and legume food samples as a function of processing conditions, without attempting to exactly mimic gastrointestinal digestion (Chapter 2). Using this method based on upper digestive tract digestion, it was observed that digestibility of the legumes increased during cooking and fermentation. Cooking improved digestibility of both soya bean and cowpea considerably (9 and 26% respectively). Subsequent fungal fermentation increased digestibility only by another 3-5% and this was influenced by fungal strain and fermentation time. Although digestibility of cooked legumes was only slightly improved by mould fermentation, the level of water-soluble dry matter of food samples increased markedly during *Rhizopus* fermentation, from an initial 7 up to 27% for soya bean and from an initial 4 up to 24% for cowpea.

Bacillus fermented legume products include among others dawadawa and soumbala made from African locust bean, and natto and kinema made from soya bean. During *Bacillus* fermentation of autoclaved soya bean for 48h, the quantity of soluble and dialysable matter increased from 22 to 65% and from 6 to 40%, respectively (Chapter 3). Protein and carbohydrate degradation during fermentation of soya bean with *Bacillus* appeared to be substantial during the first 18h of fermentation resulting in the release of high levels of peptides and oligosaccharides. *In vitro* digestibility was increased from 29% for autoclaved soya beans up to 33-43% after *Bacillus* fermentation for 48h. As for *Rhizopus* fermented soya bean (tempe), *Bacillus* fermentation of soya beans was nearly capable to reach nutrient availability to the level obtained after *in vitro* digestion of cooked soya beans, which clearly demonstrated the beneficial effect of fermentation on food nutrient availability. Fermented soya bean foods could therefore play an important role as sources of easily available nutrients especially for individuals suffering from digestive disorders.

Diarrhoea is a major problem in both human and veterinary medicine. Rotavirus and enterotoxigenic *Escherichia coli* (ETEC) are the predominant causative micro-organisms of severe diarrhoea in young children and piglets. Infection of ETEC results in high fluid secretion and electrolyte losses in the small intestine and can cause rapid dehydration and death. Minimising fluid and electrolyte losses by reducing secretion or stimulating absorption is of major importance in the treatment of ETEC diarrhoea.

Since fermented soya beans may exert an antibacterial effect, water-soluble filter-sterile extracts of tempe were tested for their ability to inhibit growth of several indicator micro-organisms including *E. coli* (Chapter 4). Antimicrobial activity was found against *Bacillus stearothermophilus* only. ETEC induced haemagglutination of hamster red blood cells was strongly inhibited by tempe extracts but hardly by a cooked soya bean extract. Furthermore, several tempe extracts were able to inhibit adhesion of ETEC to piglet small intestinal brush border membranes. Tempe appeared to interfere with *in vitro* ETEC adhesion and might therefore have a inhibitory effect on ETEC pathogenesis and resulting diarrhoea.

Oral rehydration solutions (ORSs) are used in the treatment of acute diarrhoea. Improved ORSs, in which the sodium concentration is reduced or the glucose is replaced by complex carbohydrates, have become available recently. These 'new' ORSs are thought to be beneficial primarily because of their hypotonicity (low osmolality). In this study solutions differing in composition and osmolality were used to perfuse small intestinal segments of anaesthetised piglets (Chapter 5). A strong inverse correlation between osmolality of the perfusion fluids (150-375 mOsmol/kg) and average net fluid absorption was observed both in uninfected and ETEC-infected segments. However, reducing the osmolality of the perfusion solutions below 150 mOsmol/kg hardly increased the average net fluid absorption. At low osmolality net fluid absorption was not driven by sodium absorption which suggested the existence of a physical osmotic gradient. The decrease in net fluid absorption due to ETEC infection however was independent of osmolality of the perfusion solution. The small intestinal segment perfusion (SISP) model in weaned piglets was then used to test (fractions of) fermented soya beans on their capability to decrease the observed difference in net fluid absorption between uninfected and ETEC-infected segments.

Soya bean was processed into an autoclaved, a cooked and a *Rhizopus* fermented (tempe) product. The products were pre-digested and the SISP model was used to study the effect of the products on net absorption of fluid, dry matter, sodium, chloride, potassium and total solutes (Chapter 6). All three processed soya bean products appeared to protect against the fluid loss due to ETEC infection. Cooked soya bean and tempe showed highest protection. Net fluid absorption was highest for cooked soya bean followed by autoclaved soya bean and tempe, and appeared to be related to the osmolality of the pre-digested products. ETEC infection hardly reduced net sodium and chloride absorption in segments perfused with tempe. Furthermore, in case of tempe significantly higher uptake of solutes other than sodium, chloride and potassium occurred. Therefore, processed soya bean products, particularly cooked soya bean and tempe, are beneficial in maintaining fluid balance during ETEC infection. Net fluid absorption was highest in the cooked soya bean product and was inversely correlated with the osmolality of the processed soya bean products. Tempe showed higher dry matter and total solute absorption than cooked soya bean and also sodium and chloride losses are reduced during ETEC infection. Therefore, particularly tempe may be beneficial in case of postweaning diarrhoea in piglets and possibly in children as well.

The protective effect of pre-digested tempe was compared to the effect of its constitutive fractions (Chapter 7). Tempe was pre-digested, the obtained slurry was centrifuged and the supernatant was separated by ultrafiltration using a 5kDa membrane. The SISP model was used to study the effect of the fractions on net absorption in uninfected and ETEC-infected segments. The presence of the insoluble material appeared to explain a considerable part of the protective effect of the pre-digested tempe product. However, the soluble fraction alone still showed 50% lower fluid losses compared to saline. The observed protective effect was exclusively attributable to the fraction obtained after ultrafiltration containing high-molecular-weight molecules. In addition, it was shown that pre-digestion was not a prerequisite for tempe to exert its protective effect. Tempe contains a high-molecular-weight fraction (> 5kDa) which protects against fluid loss induced by ETEC.

After having observed the positive effects of fermented soya bean *in vitro* and in the SISP experiments, it was decided to test the effects of fermented soya bean in an *in vivo* trial (Chapter 8). In a first phase piglet diet toasted full-fat soya beans (20%) were replaced with either cooked soya beans or *Rhizopus microsporus* fermented soya beans or *Bacillus subtilis* fermented soya beans. The effect on the occurrence and severity of diarrhoea in ETEC-challenged weaned piglets was determined. Severity of diarrhoea was significantly less on the diet with *Rhizopus* fermented soya beans compared with the control diet containing toasted

soya beans. Piglets fed *Rhizopus* and *Bacillus* fermented soya beans showed increased feed intake (respectively 13 and 12%), average daily weight gain (respectively 18 and 21%), and feed efficiency (respectively 3 and 8%) compared to toasted soya beans. Cooked and (especially *Rhizopus*) fermented soya beans are beneficial in the control of diarrhoea in ETEC-challenged weaned piglets and (especially *Bacillus*) fermented soya beans can be used to improve feed intake and weight gain.

Besides being nutritious and easily digestible, fermented soya bean foods could play a role in the control of ETEC-associated diarrhoea, and could therefore be particularly useful in feeding individuals suffering from diarrhoea and malnutrition.

Samenvatting

De geschiedenis van gefermenteerde voedingsmiddelen gaat duizenden jaren terug. De eerste aanwijzingen van het gebruik van fermentatie komen van de productie van bier uit gerst en wijn uit druivensap. In tegenstelling tot voedselbederf wordt fermentatie gezien als een gunstig effect van microbiële activiteit in voedingsmiddelen. Belangrijke algemene gevolgen van fermentatie zijn verlenging van de houdbaarheid (bijvoorbeeld zuurkool), verbetering van geur, smaak en textuur, en verhoging van de voedingswaarde.

Sojabonen worden al eeuwenlang gegeten in Aziatische landen. Er bestaan talloze vormen van traditionele gefermenteerde sojaproducten, zoals tempe (Indonesië) en kinema (India). Deze producten worden bereid door toepassing van zogeheten vaste-stof-fermentatie, waarbij het substraat (gekookte sojabonen) wordt omgezet (gefermenteerd) door schimmels en/of bacteriën. Het effect van dit type gefermenteerde soja op de vertering en absorptie in het maagdarmkanaal, alsmede de mogelijke gunstige effecten in relatie tot diarree, zijn onderzocht en staan beschreven in dit proefschrift.

Een *in vitro* model is ontwikkeld om het effect van verschillende bewerkingen en omstandigheden tijdens het productieproces van gefermenteerde granen en peulvruchten op de verteerbaarheid te bestuderen (Hoofdstuk 2). De nadruk ligt op het beschrijven van veranderingen als functie van de verschillende processtappen. De methode is gebaseerd op vertering in het bovenste deel van het spijsverteringskanaal (t/m de dunne darm). Koken en fermenteren van peulvruchten resulteerde in een toename van de *in vitro* verteerbaarheid. De verteerbaarheid van soja en cowpea was aanzienlijk verhoogd na koken (met respectievelijk 9 en 26%), terwijl schimmelfermentatie de verteerbaarheid vervolgens met slechts 3-5% verhoogde. De mate van veranderingen in verteerbaarheid was afhankelijk van de gebruikte schimmel en de fermentatieduur. Hoewel de verteerbaarheid van gekookte peulvruchten slechts licht verhoogd werd door schimmelfermentatie, nam het gehalte aan wateroplosbare componenten aanzienlijk toe. Fermentatie met de schimmelsoort *Rhizopus* gaf een toename van wateroplosbare droge stof van 7 tot 27% in soja en van 4 tot 24% in cowpea.

Enkele voorbeelden van peulvruchten gefermenteerd met de bacteriesoort *Bacillus* zijn dawadawa en soumbala uit Afrika en natto en kinema uit Azië. Tijdens de fermentatie van geautoclaveerde soja met *Bacillus* gedurende 48 uur namen de gehaltes aan oplosbaar en absorbeerbaar materiaal toe, respectievelijk van 22 tot 65% en van 6 tot 40% (Hoofdstuk 3). Reeds gedurende de eerste 18 uur van fermentatie werden aanzienlijke hoeveelheden eiwit en koolhydraten afgebroken tot peptiden en oligosacchariden. Echter, de verteerbaarheid steeg van 29% voor geautoclaveerde soja tot 'slechts' 33-43% na 48 uur fermentatie met *Bacillus*. Net als bij de fermentatie met de *Rhizopus* schimmel (tempe) resulteerde fermentatie met *Bacillus* in een beschikbaarheid van nutriënten vergelijkbaar met *in vitro* vertering van gekookte sojabonen. Dit duidt op een aanzienlijk gunstig effect van fermentatie op de beschikbaarheid van nutriënten. Gefermenteerde soja zou daarom dan ook een belangrijke rol kunnen spelen als bron van gemakkelijk opneembare voedingsstoffen, en is met name interessant als voedingsbron voor individuen met spijsverteringsproblemen.

Diarree is een ernstig probleem in zowel de humane als veterinaire gezondheidszorg. Rotavirus en enterotoxigene *Escherichia coli* (ETEC) zijn de meest voorkomende veroorzakers van acute diarree in jonge kinderen en biggen. Infectie met ETEC leidt tot watersecretie en het verlies van electrolyten (natrium, chloride) in de dunne darm hetgeen kan uitmonden in uitdroging en zelfs sterfte. Vermindering van water- en electrolytenverlies door

het verminderen van de secretie of het stimuleren van absorptie tijdens de infectie is van cruciaal belang in de behandeling van diarree.

Omdat gefermenteerde soja mogelijk een antibacteriële werking heeft werden wateroplosbare filtersteriele extracten van tempe getest op de mogelijkheid tot het remmen van de groei van verschillende indicator micro-organismen waaronder *E. coli* (Hoofdstuk 4). Alleen *Bacillus stearothermophilus* bleek gevoelig voor enkele extracten. Agglutinatie van rode bloedcellen door ETEC werd sterk geremd door extracten van tempe en slechts zwak door een extract van gekookte soja. Daarnaast waren verschillende tempe extracten in staat de hechting van ETEC aan darmcellen (geïsoleerd uit biggen) te remmen. Aangezien tempe de *in vitro* hechting van ETEC verminderde, zou tempe kunnen ingrijpen op de pathogenese van ETEC en mogelijk diarree kunnen voorkomen of verminderen.

Acute diarree wordt behandeld met zogenaamde 'oral rehydration solutions' (ORSs). Recent onderzoek heeft aangetoond dat verlaging van het gehalte aan natrium en/of vervanging van glucose door complexe koolhydraten resulteert in een verbeterd product. Dit is waarschijnlijk primair het gevolg van de lage osmolaliteit van deze ORSs. Perfusie van segmenten in de dunne darm van biggen (onder narcose) met oplossingen van verschillende samenstelling en osmolaliteit (150-375 mOsmol/kg) toonde een duidelijke inverse relatie tussen osmolaliteit en netto vloeistofabsorptie aan (Hoofdstuk 5). Deze relatie werd gevonden voor zowel niet-geïnfecteerde als ETEC-geïnfecteerde segmenten. ETEC infectie leidde tot een aanzienlijke reductie in netto vloeistofabsorptie onafhankelijk van de osmolaliteit van de perfusievloeistof. Het gebruikte 'dunne darm segment perfusie' of 'small intestinal segment perfusion' (SISP) model in biggen werd vervolgens gebruikt om (componenten van) gefermenteerde soja te testen op hun mogelijke beschermende werking tegen de verminderde netto vloeistofabsorptie als gevolg van ETEC infectie.

Geautoclaveerde, gekookte en gefermenteerde soja (tempe) werden voorverteerd en het SISP model werd gebruikt om het effect van deze producten op de netto absorptie van vloeistof, droge stof, electrolyten en het totaal van opgeloste stoffen te bestuderen (Hoofdstuk 6). Alledrie de behandelde sojaproducten bleken te beschermen tegen vloeistofverlies als gevolg van ETEC infectie. Gekookte soja en tempe gaven de beste bescherming. De netto vloeistofopname was het hoogste in geval van gekookte soja, gevolgd door geautoclaveerde soja en tempe, en bleek gerelateerd aan de osmolaliteit van de producten. Tempe bleek het verlies van natrium en chloride als gevolg van de ETEC infectie aanzienlijk te beperken en de absorptie van opgeloste stoffen aanzienlijk te verhogen in vergelijking met de andere producten. Daarom zou vooral tempe een positief effect kunnen hebben in geval van speendiarree en wellicht ook bij jonge kinderen met diarree.

Om de mogelijk werkzame component(en) in tempe op te sporen, werd het effect van verschillende fracties van tempe getest (Hoofdstuk 7). De tempe werd daartoe eerst voorverteerd en vervolgens werd het supernatant gescheiden van het onoplosbare deel door middel van centrifugatie. Het supernatant werd daarna gesplitst in twee fracties door middel van ultrafiltratie over een filter met een poriegrootte van 5kDa. Vervolgens werden de verschillende fracties (onoplosbaar, oplosbaar, oplosbaar > 5kDa en oplosbaar < 5kDa) getest in het SISP model om te achterhalen welke fractie de activiteit bezat. De onoplosbare stof fractie bleek een aanzienlijk deel van het beschermende effect te kunnen verklaren. Maar de oplosbare fractie bleek in staat om het vloeistofverlies als gevolg van ETEC infectie met 50% te verminderen ten opzichte van fysiologisch zout (controle). Dit effect bleek in zijn geheel afkomstig te zijn van de fractie met de bestanddelen > 5kDa en het effect bleek ook onafhankelijk van de voorvertering te zijn. Tempe bleek dus een fractie met hoog moleculaire stoffen (> 5kDa) te bevatten die beschermde tegen vloeistofverlies als gevolg van ETEC infectie.

Na de positieve effecten van gefermenteerde soja gevonden in de *in vitro* en in de SISP testen, werd het effect van gefermenteerde soja *in vivo* onderzocht. In Hoofdstuk 8 is het effect beschreven van het voeren van gekookte en gefermenteerde soja op de incidentie en de mate van diarree in pas gespeende biggen geïnfecteerd met ETEC. Biggen gevoerd met gefermenteerde soja bleken per dag gemiddeld meer te eten (±13%) en harder te groeien (±20%). Gekookte, maar vooral *Rhizopus* gefermenteerde soja (tempe), bleek effectief in het verminderen van diarree.

Gefermenteerde sojabonen zijn niet alleen licht verteerbaar en voedzaam, maar hebben ook een beschermend effect in geval van diarree en zijn daarom bij uitstek geschikt om te gebruiken bij jonge ondervoede individuen met diarree.

Curriculum vitae

Jeroen Lucas Kiers werd geboren op 3 juni 1973 te Zutphen. Na het behalen van zijn VWO-diploma in 1991 aan de Rijksscholengemeenschap Coevorden, begon hij in september van dat jaar met de studie Fysische Geografie aan de Vrije Universiteit te Amsterdam. Na het behalen van zijn propedeuse in 1992 besloot hij van studierichting te veranderen en startte met de studie Medische Biologie, tevens aan de Vrije Universiteit. Tijdens de doctoraalfase heeft hij zich gespecialiseerd in de (vrije) richting Voeding en Gezondheid. In 1995 verrichtte hij onderzoek bij de Vakgroep Fysiologie aan de Faculteit Geneeskunde van de Vrije Universiteit en in het Microbiologisch Research Laboratorium van Nutricia Research te Zoetermeer. Het laatste onderzoek tijdens zijn doctoraalfase verrichtte hij in 1996 op de afdeling Vaat- en Bindweefselonderzoek bij TNO Preventie & Gezondheid te Leiden. In augustus 1996 behaalde hij het doctoraaldiploma.

Van september 1996 tot januari 2001 deed hij een promotieonderzoek aan de Wageningen Universiteit in samenwerking met Numico Research BV en het Instituut voor Dierhouderij en Diergezondheid (ID-Lelystad). Het onderzoek, wat resulteerde in dit proefschrift, werd begeleid door Prof. Dr. Ir. F.M. Rombouts, Dr. Ir. M.J.R. Nout (Wageningen Universiteit), Dr. M.J.A. Nabuurs en Dr. J. van der Meulen (ID-Lelystad).

Sinds 1 januari 2001 is hij werkzaam als wetenschappelijk onderzoeker bij het Unilever Health Institute te Vlaardingen.

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Full papers

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Addendum

This work was carried out at Wageningen University, Department of Agrotechnology and Food Sciences, Laboratory of Food Microbiology, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, and at the Research Group Animal Physiology and Health, Division Animal Science, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands.

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Cover: Several pictures presenting an overview on most of the aspects addressed in this thesis including pictures kindly provided by Dennis Kunkel, Rob Buijs, ID-Lelystad and afdeling vakfotografie Elsevier bedrijfsinformatie bv. Design by Carolien Bartelink.