Bioactive components of fermented soya beans effective against diarrhoea-associated bacteria

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Thesis

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Abstract

Tempe is a fermented food, obtained by fungal fermentation of soya beans. During the processing specific flavour, texture and nutritional properties are achieved. Previous research has indicated that tempe reduced the incidence and severity of diarrhoea. In this thesis the bioactive effects of tempe on diarrhoea-associated bacteria are described.
Tempe appeared to be antibacterial against *Bacillus cereus* cells and spores, but not against enterotoxigenic *Escherichia coli* (ETEC). Nevertheless, tempe was found to inhibit the adhesion of ETEC to intestinal epithelial cells.
Experiments showed that the inhibition of adhesion was caused by an interaction between ETEC and tempe extracts. A range of ETEC strains was shown to be sensitive for the anti-adhesive component, making the bioactivity of broader interest for applications in feed matrices.
Furthermore, several substrates (legumes and cereals) were fermented and all fermented legumes tested were equally bioactive as the fermented soya beans, whereas the fermented cereals were not active. The use of different starter cultures showed that *Bacillus* spp., moulds and some yeasts were capable to release or form the bioactive component during fermentation, thus the bioactivity was not specific for one microbial species.
After heating, defatting and protease treatment of the bioactive tempe extracts, they remained bioactive. On the contrary, after treatment with polysaccharide degrading enzyme mixtures the bioactivity was lost. This suggests that the bioactive component contains carbohydrates, and explains the interaction between ETEC and tempe extracts, which could indeed be established by carbohydrates of the tempe extract. Ultra-filtration revealed the bioactive component to have molecular masses >30 kDa. Further purification yielded an active fraction with an increased carbohydrate content. Monosaccharide analysis showed the importance of arabinose in the bioactive components.
In conclusion, the bioactive component is released or formed during fermentation by enzymatic degradation of legumes. The bioactive component is of carbohydrate nature and contains arabinose, which originates from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of legumes.

Contents

Chapter 1

Introduction

Fermented foods

Fermentation is defined as bioprocessing using microorganisms and their enzymes to achieve desirable quality characteristics of food products. Both plant and animal ingredients can be fermented and also the microorganisms used can belong to diverse groups, namely bacteria, yeasts and filamentous fungi (moulds) (Nout *et al.*, 2005).

The origin of fermented foods goes back many thousands of years and fermentation is one of the oldest ways of food processing. Popular fermented products, such as beer, bread, wine and sausages, have been around for centuries (Campbell-Platt, 2004; Nout *et al.*, 2005). Other examples of popular fermented food products are yoghurt, cheese, coffee, tea, alcoholic drinks, soy sauce, sauerkraut and tempe. Fermentation can have diverse roles: (a) enrichment of the diet, through development of a diversity of flavour and texture in food substrates, (b) preservation through alcoholic, lactic acid, acetic acid and alkaline fermentation, (c) nutritional enrichment of the food with vitamins, essential amino acids or fatty acids, (d) detoxification and removal of anti-nutritional factors, and (e) decreased cooking times (Steinkraus, 1996).

Tempe

Tempe is a collective name for cooked and fermented beans, cereals or food-processing by-products, penetrated and bound together by the mycelium of a living mould. Yellow-seeded soya beans are the most common and preferred raw material to produce tempe (Nout and Kiers, 2005).

Figure 1: Tempe, as sold in the Netherlands.

Tempe originates from Indonesia, the authentic Indonesian spelling is “tempe”, whereas the spelling “tempeh” is also used in literature. In this thesis the authentic Indonesian spelling will be used. Tempe is pronounced as TEM-pay (Shurtleff and Aoyagi, 2001). In Indonesia, tempe is consumed as a protein-rich meat substitute by all economic groups. Outside Indonesia, tempe gains interest as a major protein source other than meat (Steinkraus, 1996; Astuti, 2000; Nout and Kiers, 2005).
Figure 1 shows soya bean tempe manufactured and sold in the Netherlands. The mould grows not only on the surface of the bean cake, but throughout the bean mass, knitting the soya beans into a compact cake.

The origin and history of tempe

Tempe is unique among major traditional soya foods, because it is the only fermented soya food product that did not originate in China or Japan (Shurtleff and Aoyagi, 2007). Tempe originates from Central and East Java in today’s Indonesia. The earliest references in Indonesian literature are from the early 1800s, but tempe is believed to have evolved long before that time.
As Indonesia has been a Dutch colony for centuries since the late 1600s, some early research findings were published by Dutch scientists. In 1875 the term tempe was defined in the Javanese-Dutch dictionary as “fermented soybeans or press-cake baked or fried in flat pressed cakes. It is well liked as a side dish with rice”. In 1895 the Dutch microbiologist and chemist Prinsen Geerlings made the first attempt to identify the tempe mould (Shurtleff and Aoyagi, 2007). Up till now many publications have dealt with microbiological, biochemical and nutritional changes during the tempe fermentation. Also different books (chapters) and reviews about tempe have been published (Ko and Hesseltine, 1979; Nout and Rombouts, 1990; Steinkraus, 1996; Shurtleff and Aoyagi, 2001; Tibbott, 2004; Nout and Kiers, 2005). For many decades tempe has been regarded as a meat alternative for poor communities because of its high protein content. As a result of the low-cost technology available for processing this food, its low price, and its nutritional value, tempe is a traditional food consumed by indigenous Indonesians (Karyadi and Lukito, 1996).
Nowadays, tempe obtains its popularity from its non-meat protein-rich nature, nutritional and health functionality. Figure 2 shows the main reasons why tempe is an ideal food for use in developing countries as a source of tasty and inexpensive high-quality protein (Shurtleff and Aoyagi, 2001).

1. Production requires only simple low-level technology with low costs.
2. The only ingredients are soya beans or other raw material (i.e. legumes, grains) including waste products, water and a starter.
3. The warm or tropical climates characteristic of so many developing countries greatly facilitate the tempe fermentation.
4. The fermentation is unusually simple and short (24 to 48 hours) as compared with several months for many other fermented foods.
5. Tempe has a taste and texture, appearance and aroma that are well suited to use in local cuisines.
6. Tempe is an ideal meat substitute, healthy, tasty and easy to digest.

Figure 2: Characteristics of tempe as an ideal food in developing countries (Adapted from: Shurtleff and Aoyagi 2001).

Production of tempe

Yellow-seeded soya beans are the most common and preferred raw material for tempe. Nevertheless, other substrates such as barley (Eklund-Jonsson *et al.*, 2006; Feng *et al.*, 2007), chick pea (Ashenafi and Busse, 1991), cowpea (Kiers *et al.*, 2000a; Egounlety, 2001), groundbean (Egounlety, 2001), horse bean (Ashenafi and Busse, 1991), lima bean (Ko and Hesseltine, 1979), pea (Ashenafi and Busse, 1991), oats (Eklund-Jonsson *et al.*, 2006), sorghum (Mugula and Lyimo, 2000) and wheat (Hachmeister and Fung, 1993) have been reported to be suitable substrates. Some substrates can only be processed by combining them with soya beans. Also mixtures of legumes with non-legumes, and other plant materials, such as apricot seeds or maize or food-processing by products, can be used in the tempe fermentation (Nout and Kiers, 2005; Feng, 2006).

The main process operations that differ according to the use of various substrates used in tempe fermentation are the selection of optimum pre-treatments such as de-hulling, optimum soaking conditions or duration of boiling. The process of tempe manufacture from soya beans is shown in figure 3. The process starts with the de-hulling of the soya beans, which can be done manually by rubbing the seed coats from soaked soya beans, or by mechanical abrasion of dry beans. In the Netherlands, tempe manufacturers purchase dry de-hulled soya beans, ready for use. The soya beans are soaked for 6-24 h, in order to: (1) increase the moisture content of the beans, (2) to enable microbial

activity in the soaking water, (3) render the beans edible and (4) to extract naturally occurring antimicrobial substances (saponins) and bitter components.
Some manufacturers add lactic or acetic acid or naturally acidified soaking water "backslop" (Nout *et al.*, 1987) at the start of the soaking, to control microbial spoilage.

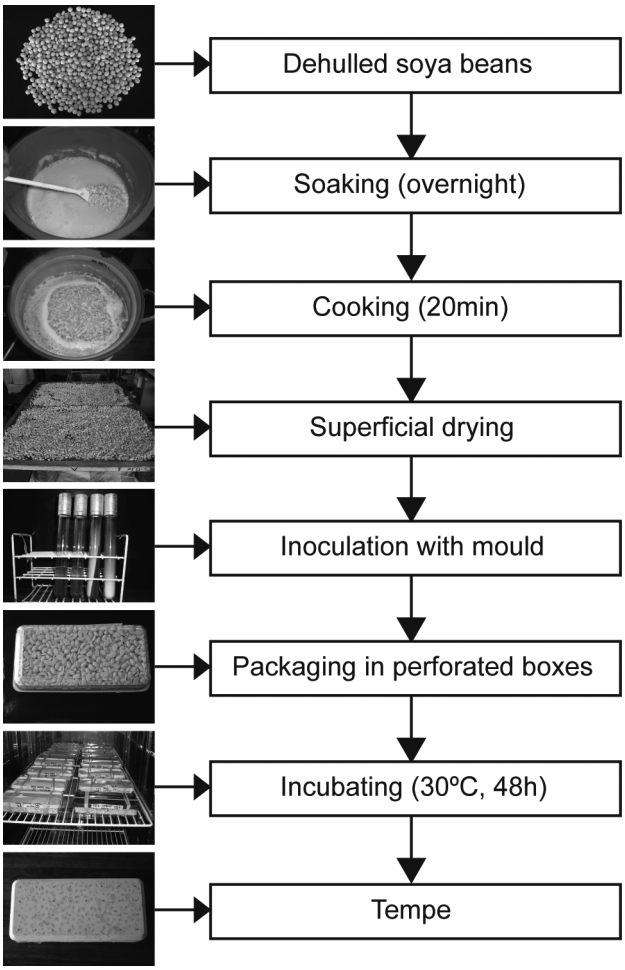

Figure 3: Production process of soya bean tempe.

Subsequently, the soaking water is discarded and the beans are cooked for 20-30 minutes in fresh water. After cooking, the cooking water is discarded and the beans are spread out on perforated trays to remove free water, steam-off and achieve a rapid

cooling. When working at a large scale, basket centrifuges are used to remove the cooking water. The cooled soya beans are inoculated using a tempe starter containing sporangiospores of mainly *Rhizopus* spp., and sometimes *Mucor* spp. with a concentration of about 10^4 colony-forming units (CFU) g^{-1}. Traditionally, the beans are packed in punctured banana leaves allowing a limited supply of air to the beans (figure 4).

Figure 4: Fresh tempe at the market, Jakarta, Indonesia (Photo taken by: Sakurai Midori).

Nowadays, flexible plastic bags, tubing (sausage casings) or hard plastic boxes with adequate perforation openings, to allow aerobic growth of the mould, are in use. The inoculated and packed beans are incubated for 1-2 days at 25-30ºC. Due to the restricted air supply, the formation of fungal sporangiospores is restricted, resulting in an attractive creamy, white fresh tempe cake. Fresh tempe is not eaten raw, but first cooked or fried and used in a variety of dishes. The traditional and modern tempe processing has been reviewed extensively (Steinkraus, 1985; Nout and Rombouts, 1990; Steinkraus, 1996; Shurtleff and Aoyagi, 2001; Nout and Kiers, 2005).

Microbiological composition of tempe

The microflora in tempe is complex, as tempe is a result of a mixed culture fermentation by moulds, yeasts, lactic acid bacteria and various other bacteria. The

major genus of importance is the mould *Rhizopus* with different species such as *R. microsporus, R. oligosporus* and *R. oryzae* (Nout and Kiers, 2005).
Lactic acid bacteria play a role in the acidification of the soya beans during soaking, thereby preventing the growth of spoilage microorganisms (Nout *et al.*, 1987; Ashenafi and Busse, 1991) and thus improving the shelf life of tempe. During fermentation, lactic acid bacteria grow up to 10^9 CFU g^{-1} in final tempe products.
The microbial quality of 110 samples of commercial tempe in the Netherlands was studied and it was shown that most had an aerobic plate count exceeding 10^7 CFU g^{-1}, with high numbers of Enterobacteriaceae and lactic acid bacteria. Yeast levels higher than 10^5 CFU g^{-1} were found in 69% of the samples and some also contained *Staphylococcus aureus*, *Bacillus cereus* or *Escherichia coli* (Samson *et al.*, 1987). Also Ashenafi (1994) found high numbers of enterobacteria, enterococci and staphylococci, whereas Mulyowidarso *et al.* (1990) found high numbers of *Bacillus* species in tempe.
The contribution of bacteria and yeasts to the properties of tempe is only partly understood, but they can play a role in flavour development and substrate modification, and in the safety of the product (Nout and Rombouts, 1990).

Biochemical changes occurring during fermentation
During fermentation of soya beans several biochemical changes take place, which enhance the nutritional and sensory quality of the tempe. This is mainly due to the activity of the fungal enzymes. The mould, *Rhizopus* spp. produces a variety of carbohydrases, lipases and proteases, which degrade the macronutrients into lower molecular substances with a higher water-solubility. Also vitamins, phytochemicals and anti-oxidative constituents are formed (Astuti, 2000; Nout and Kiers, 2005).
Table 1 shows a compilation of published data concerning the composition of cooked soya and tempe. Different varieties of soya beans and other processing parameters can influence the composition. During fermentation only small changes in total crude protein, crude lipid and total carbohydrates were reported.
Whereas the change in total nitrogen content during fermentation is negligible, an increase of free amino acids takes place, due to hydrolysis of the proteins. The major soya proteins are glycinin and β-conglycinin. β-Conglycinin is more sensitive to protease activity than glycinin, which can be related to its chemical structure (Nowak, 1992; De Reu *et al.*, 1995). The degree of hydrolysis strongly depends on the fungal strain and the fermentation conditions.
The amount of fatty acids present in triglycerides decreased during fermentation, whereas the free fatty acids increased in the final product, but *Rhizopus* is also using

part of the fatty acids as a carbon source (De Reu *et al.*, 1994). The production of only small amounts of glycerol indicates that triglycerides were primarily hydrolysed to mono- and diglycerides and free fatty acids (Ruiz-Terán and Owens, 1996).

Table 1: Composition of cooked soya beans and tempe.

	Cooked soya (100g)		Tempe fresh (100g)				
Reference	2	3	1	2	3	4	5
Energy (kJ)	590	624	657	808	691	603	
Moisture (g)	69		60	60		72	64
Protein (g)	12	14.3	20	19	15.7	12	18
Crude lipids (g)	6	7.7	8	11	6.4	8	4
Carbohydrates (by difference) (g)	11	8.5	10	9	14.1	6	13

1. (Shurtleff and Aoyagi, 2001)
2. (USDA, 2009)
3. (USB, 2010)
4. (Voedingscentrum, 2006)
5. (Voedingswaardetabel, 2004)

Carbohydrates in soya beans comprise mainly cell wall polysaccharides and the small sugars fructose, raffinose and stachyose. These small sugars are removed during soaking, cooking and fermentation of the soya beans (Mulyowidarso *et al.*, 1991; Egounlety and Aworh, 2003). The insoluble cell wall polysaccharides, such as pectin, cellulose and hemicellulose are (partly) degraded during fermentation by the enzymes of the mould which leads to their enhanced water-solubility (Kiers *et al.*, 2000a). The major monosaccharide constituents in soya bean cell walls are galactose, glucose, arabinose and galacturonic acid (Huisman *et al.*, 1998). The major carbohydrases of *R. oligosprorus* in tempe were reported to include polygalacturonases, cellulases, xylanases and arabinanases (Sarrette *et al.*, 1992). Soya bean pectin consists of two types of backbones, namely a polygalacturonic acid (1,4)-backbone and a rhamnogalacturonan backbone. The rhamnogalacturonan backbone is substituted with polymers like arabinans, galactans or arabinogalactans. During fermentation of soya beans the pectin fraction and its arabinogalactan side chains are predominantly solubilised (De Reu *et al.*, 1997).

The anti-nutritional factors (ANF), such as trypsin inhibitors and lectins, are mainly leached out or inactivated during soaking, cooking and fermentation. The decrease of

phytic acid is very important, because it binds to minerals, thereby lowering the mineral bioavailability (Prinyawiwatkul *et al.*, 1996; Tawali *et al.*, 1998; Astuti, 2000; Egounlety and Aworh, 2003; Nout and Kiers, 2005). The levels of some vitamins of the B group, especially riboflavin, niacin, vitamin B6 and vitamin B12, increase during fermentation, because of fungal and bacterial metabolic activities (Bisping *et al.*, 1993; Keuth and Bisping, 1993; Denter *et al.*, 1998).

Soya beans and soya products contain three isoflavones, genistein, daidzein and glycitein and also various saponins. The concentrations of isoflavones and saponins vary according to soya bean varieties, growing location, cultivation year and degree of maturity (Hubert *et al.*, 2005). Processing of soya beans can result in losses of some isoflavones, especially during soaking and cooking. Fermentation was reported not to cause a significant loss of isoflavones, but aglycons are released from the glucosides by the action of β-glucosidase (Wang and Murphy, 1996; Murphy *et al.*, 1999). Research on saponins during processing of soya also showed enzymatic deglycosylation and some losses during cooking and soaking, but data are still limited (Hubert *et al.*, 2005; Sinha *et al.*, 2005).

Health aspects of tempe

The effects of soya beans on the health of man and animals has been the subject of several studies. Indeed a number of beneficial effects were reported. Despite the large number of studies that have been performed, many conflicting data have been found, especially in relation to the prevention of chronic diseases (Messina *et al.*, 2002; Balk *et al.*, 2005). Soya is associated with beneficial health effects on cardiovascular diseases, menopausal symptoms, endocrine function, cancer, bone health, reproductive health, kidney diseases, cognitive function and glucose metabolism. Many of the health aspects are related to the phytochemicals present, such as isoflavones and saponins. Only one health claim for use on food labels was approved by the U.S. Food and Drug Administration in 1999. This claim is: ”The inclusion of 25 g soya protein per day in a diet low in saturated fat and cholesterol, may reduce the risk of heart disease” (FDA, 1999). Carefully controlled efficacy studies may still be useful to pin down the relative effects of various components of soya (Balk *et al.*, 2005).

Whereas soya beans are the main ingredient of tempe and the health effects of soya beans can also be associated with tempe, tempe is associated with certain specific health effects.

After fermentation, the absorption and digestibility of the soya beans increase which can have beneficial physiological effects in case of malfunction of the gastrointestinal

digestive system (Kiers *et al.*, 2000a). The high digestibility of tempe was already observed during World War II when prisoners suffering from dysentery were able to digest tempe much better than soya beans (Steinkraus, 1996; Tibbott, 2004).
Karyadi and Lukito (1996) described studies performed in Indonesia on the hypolipidemic properties of tempe. In a number of clinical intervention trials, total cholesterol and low-density lipoprotein cholesterol were significantly reduced in persons treated with tempe, whereas HDL cholesterol was raised (Karyadi and Lukito, 1996; Astuti, 2000; Karyadi and Lukito, 2000). Soya beans contain natural antioxidants and during fermentation the anti-oxidative capacity increases (Berghofer *et al.*, 1998; Chen-Tien *et al.*, 2009). Furthermore, several studies demonstrated an anti-diarrhoeal effect of tempe. The next paragraph will deal with the latter aspect in more detail.

Tempe and diarrhoea
In the early 1960s, tempe was reported to contain an antibacterial substance, acting especially against a number of Gram-positive bacteria i.e *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus* and *Clostridium* spp. (Wang *et al.*, 1969; Wang *et al.*, 1972; Kobayasi *et al.*, 1992). These studies suggest the presence of a component from the tempe or the *Rhizopus* spp, that inhibits the growth of these bacteria.
Other researchers investigated the role of tempe, as part of the diet, in the development of diarrhoeal infection in animals (table 2a) and humans (table 2b). Most of this work has been done in Indonesia and not all is available in English literature, but all studies published in English are presented in table 2.
Table 2a shows the research on the effects of tempe on diarrhoea in piglets (Kiers *et al.*, 2003) and rabbits (Karmini *et al.*, 1997). Both were infected with an *E. coli* strain and both showed a lower severity of diarrhoea, when fed with tempe. Table 2b shows four studies of the effect of tempe addition to a human diet, on the severity of diarrhoea. All studies gave similar results, namely a shorter duration of diarrhoea, when tempe was consumed. The data about the specific health effects of tempe is especially of interest in patients suffering from intestinal digestive defects. Tempe-based food could play a role as a source of easily available nutrients for individuals suffering from malnutrition and/or acute diarrhoea for whom the need of easily digestible rehabilitation foods is high. The anti-diarrhoeal properties of the product make it even more attractive in the prevention and management of malnutrition (Nout and Kiers, 2005). In countries with malnutrition, tempe-based weaning food can be helpful. Research showed that there is a potential for using tempe-based formulas in weaning diets (Osundahunsi and Aworh, 2002).

Table 2a: Effects of tempe on *E. coli* infection in animals.

References	Target group	Treatment	Microorganism	Exposure time	Main results
Karmini *et al.*, (1997)	6-week old male rabbits (n=84)	Tempeh based formulated food (TF), soya bean-based formulated food (SF), milk-based formulated food (MF) and formulated food without protein (FO)	EPEC O125:K70(B)H19 on 4 consecutive days	2 weeks	Onset of diarrhoea: TF 5.07, SF 4.0, MF 3.64 PF 2.36 days. Diarrhoea occurred in 36% of rabbits in the TF group and in 50-64% in the other groups
Kiers *et al.,* (2003)	4-week old piglets (n=96)	Cooked soya (CS), tempe (T), *Bacillus*-fermented soya beans (BT), de-hulled full-fat toasted soya beans (TS)	ETEC O149:K91:K88^{ac} on day 1 of the experiment	4 weeks	Diarrhoea incidence: CS 37, T 33, BT 38, TS 46%, diarrhoea severity: CS 1.9 T 1.7, BT 1.8, TS 2.3, days with diarrhoea CS 5.0, T 4.3, BT 4.8, TS 6.2 days

Table 2b: Effects of tempe on human diarrhoea patients.

References	Target group	Treatment	Dosis	Main results
Kalavi *et al.,* (1996)	Protein-energy malnourished children 6-60 months (n=117)	Milk-yellow maize porridge (MYMP) (n=61) and tempe-yellow maize porridge (TYMP) (n=56)	*Ad libitum* during 1 month	Duration of diarrhoea (days) MYMP 4.6, TYMP 0.7
Mahmud *et al.,* (1985)	Children <5 years (n=111) with chronic diarrhoea	Tempeh-based formula (n=79) or milk based infant formula (n=32)	Supplementary during diarrhoeal episode	Duration of diarrhoea (days) tempeh-based food mixture 2.39, milk-based formula 2.94
Partawihardja, (1990) in Karyadi and Lukito, (1996)	Children aged 6-24 months with acute diarrhoea (n=304)	Formulated food without tempeh (A1) (n=75), tempeh formulated food (A2) (n=81), tempeh powder (A3) (n=75) and homemade food (A4) (n=73)	Supplementary during diarrhoeal episode	Duration of diarrhoea (days) A1 6.36, A2 4.83, A3 5.13 , A4 5.83 days
Soenarto *et al.,* (1997)	Children aged 6-24 months with acute diarrhoea (n=214)	Traditionally produced tempe-based formula (TT) (n=72), industrially produced tempeh (IT) (n=72), soya bean powder formulated foods (IS) (n=68)	Starting in hospital and continued at home for up to 90 days after hospitalization; supplementary 2 sachets of formula daily	Duration of diarrhoea (days) TT 3.4, IT 3.5, IS 3.9

Pathogenesis of bacterial diarrhoea

In this paragraph the pathogenesis (the mechanism by which a disease is caused) of bacterial diarrhoea is described. Diarrhoea is defined as the passage of three or more loose or liquid stools per day. Diarrhoeal disease is worldwide the second leading cause of death in children under five years old, and is responsible for killing 1.5 million children every year. Diarrhoea is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms (WHO, 2009). This thesis focuses on the influence of tempe on bacterial diarrhoea, because previous research has shown the beneficial effect of tempe against diarrhoea caused by bacterial infection (Kiers *et al.*, 2003).

Bacteria have one or more special abilities, such as colonization, adhesion, invasion or toxin production that contribute to the pathogenicity. When the bacteria are able to grow within the host cells this is referred to as infection. Next to infections, bacterial diarrhoea can be caused by intoxications. Intoxication is a disease that results from the uptake of a specific toxin (which can be produced by a microorganism) into the body of a host. Toxins can even induce disease in the absence of the producing organism. Bacterial toxins are distinguished into two groups according to their chemical properties: (a) exotoxins, which are soluble proteins in cell extracts or in the growth medium; (b) endotoxins, which are the lipopolysaccharide components of the outer membranes of Gram-negative bacteria. Bacterial toxins are highly specific and target the host in different ways.

Various bacteria have their own typical way to infect the host. Basically infections develop in the following steps:

1. Maintain a reservoir of the bacterial pathogen

All bacterial pathogens must have a reservoir where they can survive, either in humans, animals or the environment.

2. Transport of the bacterial pathogen to the host

Bacteria can be transmitted directly from host to host by coughing, sneezing and bodily contact. But also indirectly, infected hosts shed bacteria into the surroundings, soil, water and food and transmit them in this way to a new host.

3. Adhere to, colonize and/or invade the host

To initiate pathogenesis, bacteria must be able to colonize a surface or invade a tissue. In order to colonize mucous membranes of the gastro-intestinal tract, bacteria must adhere to mucosal cells, because the surfaces of these membranes are recurrently washed with fluids that sweep away non-adhered organisms. Bacterial cells can adhere to particular tissues in a specific manner. The adherence factors (adhesins), are

specialized molecules or structures on the bacterial cell surface that bind to complementary receptor sites on the host cell surface. Examples of adhesins are the capsule, the lipopolysaccharide layer, certain outer membranes and fimbriae. Bacteria can also adhere in a non-specific manner to the host, i.e. by hydrophobic interactions, electrostatic attractions, atomic and molecular vibrations, Brownian movement and recruitment and entrapped in biofilms.

Entry into host cells and tissues is a specialized strategy used by many bacterial pathogens for survival and multiplication. Invasion of the host by a pathogen may be aided by the production of bacterial extracellular substances (invasins). Most invasins are proteins (enzymes) that cause local damage to host cells and/or have the immediate effect of facilitating the growth and spread of the pathogen. The damage to the host as a result of this invasive activity may become part of the pathology of an infectious disease.

4. Growth and multiplication of the bacterial pathogen

For the growth of the pathogens an appropriate environment with adequate nutrients, favourable pH, temperature and redox potential is needed. Some bacteria invade specific cells in which they grow and multiply to cause an infection.

5. Initially evade host defense mechanism

Pathogens have special properties that protect them from elimination by host defenses. Many pathogens produce a capsule that confers resistance to phagocytosis. Also, specific proteins on the surface of pathogens have been shown to confer resistance. Other pathogens resist phagocytosis by secreting proteins or chemical substances that interfere with host cell functions or with phagocytes. Some pathogens can even grow inside phagocytes.

6. Posses the ability to damage the host

Pathogens can cause infectious disease resulting from the pathogen's growth and reproduction that often produce tissue alteration.

7. Leave the host and return to the reservoir or enter a new host

A successful bacterial pathogen is able to leave the host and enter either a new host or a reservoir. Most bacteria use passive escape mechanisms, such as faeces, urine, saliva or flakes of skin cells (Stanier *et al.*, 1993; Prescott *et al.*, 2002; Todar, 2009).

In this thesis most of the research was done with Enterotoxigenic *Escherichia coli* (ETEC) and *Bacillus cereus*. The specific pathogenicity of these two bacteria are discussed in the next paragraphs.

Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) is defined as *E. coli* that elaborate at least one of the two enterotoxins, i.e. heat-stable toxin (ST) and heat labile-toxin (LT). ETEC is a global cause of severe, watery diarrhoea in the offspring of some animal species such as newborn (suckling) calves and suckling and weaned pigs (Nagy and Fekete, 1999). In humans, ETEC is recognised as one of the most frequent causes of childhood diarrhoea in developing countries. Also, it is an important causative agent of traveller's diarrhoea (Dalton *et al.*, 1999; Bhan, 2000). Many similarities can be found in the pathogenisis by ETEC infections of animals and humans which provides opportunities to understand human ETEC infections by the use of animal models (Nagy and Fekete, 2005).

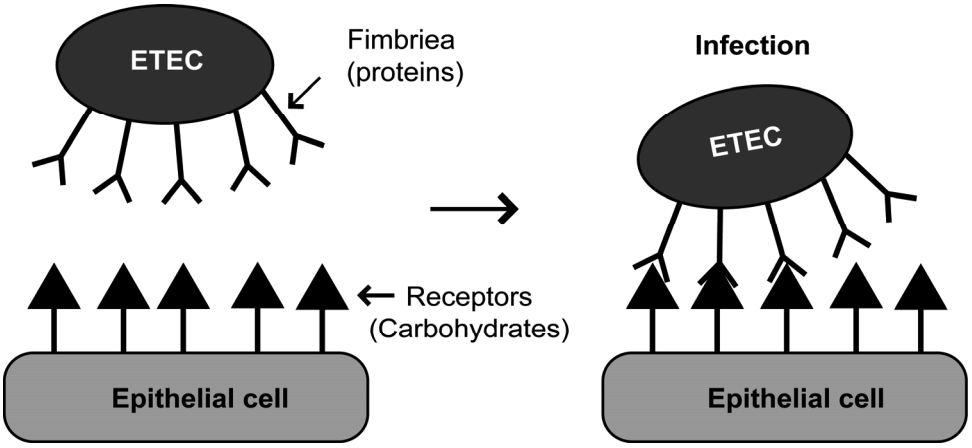

Figure 5: Adhesin-ligand interaction of bacteria and the host.

The first step in the infection by ETEC is the adhesion and colonization of the intestinal mucosa without inducing significant morphological changes. This adhesion is mostly caused by a specific adhesion-ligand interaction (figure 5). Fimbriae are the most common adhesive surface antigens of ETEC. They are called colonization factors and are made up of proteins (Valvatne, 2004). ETEC strains expressing K88 (fimbriae, that are designated as K antigens) are only virulent for pigs, whereas K99-expressing organisms are pathogenic for calves, lambs and pigs (Nataro and Kaper, 1998). The colonization factors are nowadays classified by F numbers, F4 an F5 for K88 and K99, respectively. Because of the specificity of these adhesins, animal ETEC strains normally do not infect humans (Qadri *et al.*, 2005).

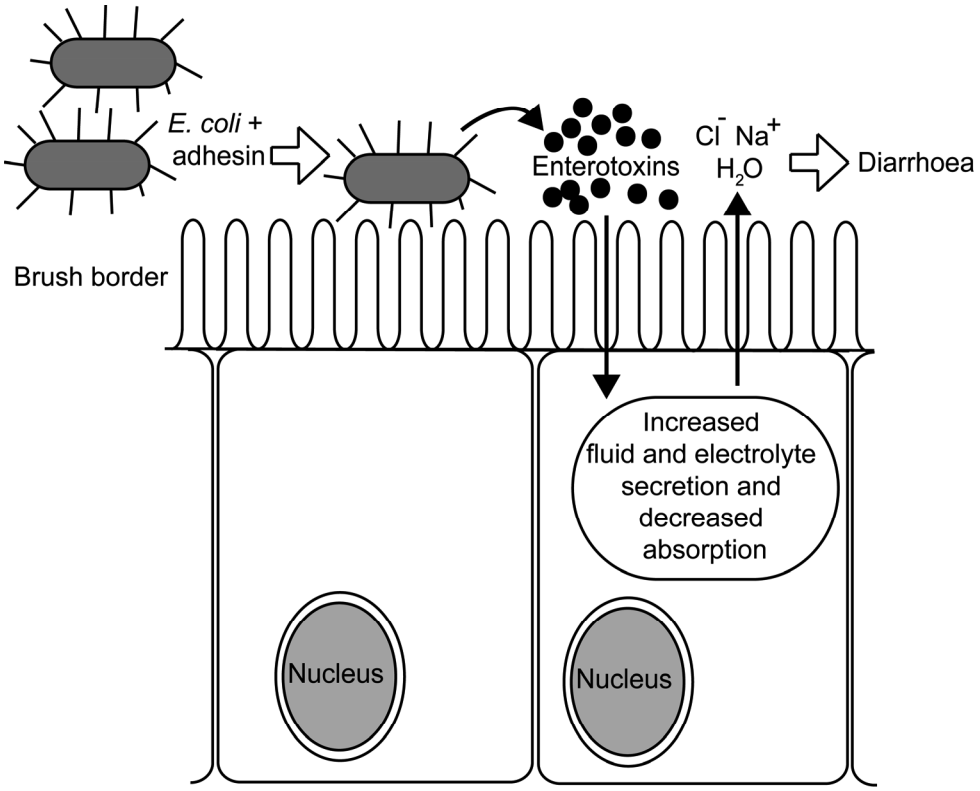

Figure 6: Pathogenisis by ETEC (partly adapted from Evans and Evans, (1996)).

Human colonization factors are named colonization factor antigen (CFA), coli surface antigen (CS) and putative colonization factor (PCF). More than 20 colonization factors have been identified and characterized. They are subdivided by their antigenicity, molecular weight, N-terminal amino acid sequence of the major subunit and structural morphology as either fimbrial, fibrillar, helical or non-fimbrial (Turner *et al.*, 2006). The nomenclature to classify is designating as coli surface antigens (CS) with a number corresponding to the chronological order of identification with the exception of CFA/I (Gaastra and Svennerholm, 1996). There are also a number of less well-characterized colonization factors, and new ones are likely to be identified in the future (Turner *et al.*, 2006).

When close contact is established between the ETEC and the host cells, ETEC can excrete one or more enterotoxins (belonging to the exotoxins). Figure 6 illustrates the pathogenesis by ETEC. The basic mechanism of action of the heat-labile (LT) and

heat-stable (ST) enterotoxins is that they cause profound functional changes in small intestinal epithelial cells, resulting in increased secretion of H_2O, Na^+ and Cl^- and a concomitant decrease of fluid absorption, leading to dehydration and acidosis. The mechanisms of action and structure of LT enterotoxin are very well established and are very similar to those of the heat-labile toxins of *V. cholerae*. LT enterotoxin 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.
Two types of heat-stable toxins are produced by ETEC, STa and STb. The biological activity of STa is exerted through stimulation of the guanylate cyclase system, leading to an increased intracellular cyclic guanoside monophosphate (cGMP) and reduced absorption of water and electrolytes. STb is mostly associated with porcine strains of ETEC, but has also been found in humans. The mechanism of action and the molecular characteristics are still much less known than of STa (Nagy and Fekete, 2005). The toxic effects are characteristic of ETEC infection and lead directly to the manifestation of secretory diarrhoea (Turner *et al.*, 2006).

Bacillus cereus

Bacillus cereus is a Gram-positive, spore-forming bacterium that can cause a diarrhoeal or emetic syndrome. *B. cereus* is very common in soil, because of its low nutrient requirements. Due to this property and the ability to form spores, *B. cereus* has the capacity to spread easily.
The diarrhoeal syndrome is encountered by heat-labile enterotoxins, which are produced during the vegetative growth of *B. cereus* in the small intestine. The emetic syndrome is caused by a heat-stable toxin, which is produced by growing cells in foods prior to consumption (Kotiranta *et al.*, 2000).
The prevalence of *B. cereus* food-borne illnesses is difficult to determine, because the symptoms associated with infection or intoxication are generally mild. Conceivably, most of the cases are not reported and prevalence is underestimated. Despite this underestimation *B. cereus* was the causative agent in food-borne illnesses in 13% to 43% of all reported incidents with a known cause in The Netherlands, between 2004-2007 (Doorduyn *et al.*, 2009).
Products that can cause *B. cereus* infection are boiled and baked rice, flour products, meat, vegetables and milk products (Schoeni and Wong, 2005).

B. cereus can also form biofilms by attaching to processing equipment and surfaces, especially those that are in contact with food. Bacterial cells that are embedded in a biofilm tend to be more resistant to cleaning agents, making them hard to eradicate (Peng *et al.*, 2002). The economic impact of *B. cereus* is huge, because of the growth, biofilm and spore-forming in food and food processing.

Background of the thesis

The fact that diarrhoea is a major health problem worldwide in children as well as in farm animals, underlines the importance of the research for anti-diarrhoeal agents. Tempe was found to improve the recovery from diarrhoea in children when it was consumed as an ingredient of the infant food formula (Mahmud *et al.*, 1985; Kalavi *et al.*, 1996; Soenarto *et al.*, 1997).

Previous research in our laboratory was directed towards the effects of fermented soya bean foods on digestion, absorption and ETEC-induced diarrhoea in the gastro-intestinal tract (Kiers, 2001). It was observed that digestibility of legumes increased during cooking and fermentation and the water-soluble dry matter increased markedly during *Rhizopus* fermentation (Kiers *et al.*, 2000a). Also, during *Bacillus* fermentation an increase in soluble and dialysable material was observed as well as major degradation of polymers (Kiers *et al.*, 2000b). Tempe was reported to inhibit adhesion of ETEC to piglet intestinal brush border cells (Kiers *et al.*, 2002). Adhesion of ETEC to intestinal cells is the first step in the pathogenisis of these bacteria, thus the inhibition of ETEC adhesion is crucial in preventing infection. However, the mode of action, i.e. how tempe could inhibit the adhesion of ETEC to intestinal cells, remained unclear.

In those studies, the small intestinal segment perfusion model in weaned piglets was used to test soya bean products for their capability to decrease the difference in net fluid absorption between uninfected an ETEC-infected segments. Soya bean products (autoclaved, cooked and fermented) protected against fluid loss. For tempe, significantly higher uptake of solutes occurred. Particularly tempe may be beneficial in case of post-weaning diarrhoea in piglets (Kiers *et al.*, 2006). This protective effect was exclusively attributed to the fraction obtained after ultrafiltration containing high-molecular-mass molecules (>5 kDa) (Kiers *et al.*, 2007). In an *in vivo* piglet trial the severity of diarrhoea was significantly less for pigs fed a diet containing *Rhizopus* fermented soya bean compared with pigs fed the control diet containing toasted soya beans (Kiers *et al.*, 2003).

Nowadays, in piglet diets, carbohydrases are used to improve nutrient utilization. It was reported that the addition of carbohydrases to piglet diets reduced the frequency and severity of diarrhoea (Partridge, 2001). Another research showed that the hydrolysis products of non-starch polysaccharides of soya bean meal were beneficial in fluid balance during ETEC infection (Kiarie *et al.*, 2008). In tempe, the mould enzymes are actively degrading the macromolecules, which seems to be an important factor to increase the anti-diarrhoeal effect of soya beans. Investigations are required into the chemical characteristics of the protective component(s) in tempe, since this would be of interest for the prevention of diarrhoea in animals as well in humans.

Aim and outline of the thesis

This thesis deals with the different bioactive effects of tempe on diarrhoeal bacteria. The research was performed to investigate the anti-diarrhoeal effect of tempe on diarrhoea-associated bacteria in more detail. Research was performed to understand the mode of action and elucidate the specificity of the anti-diarrhoeal effect. In addition, chemical analyses were performed to elucidate the composition of the chemical substance responsible for the bioactivity of tempe.

This **chapter 1** provides an introduction to the thesis and offers general information about tempe and the fermentation process. Next, an overview of the health aspects that are associated with tempe is provided with special reference to the impact on diarrhoea. In **chapter 2**, the effect of tempe on the growth of ETEC and its adhesion to intestinal cells is dealt with. The effect of tempe extracts on the growth of *Bacillus cereus* cells and spores is described in **chapter 3**. In **chapter 4** the adhesion inhibition effect of tempe towards different ETEC strains is studied as well as the bioactivity of tempe produced with different raw materials and different starter pure cultures. In **chapter 5** the chemical nature of the adhesion inhibiting component of tempe is studied**.** Finally in **chapter 6** a general discussion is presented.

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Chapter 2

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

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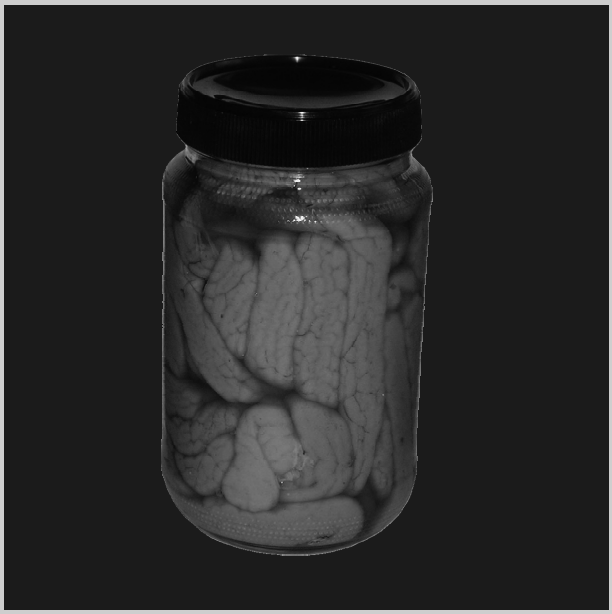

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Abstract

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

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

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

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

Introduction

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

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

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

Kiers *et al.* (2002) focused on ETEC adhesion to piglet brush border cells, as affected by the final product of soya bean fermentation (tempe). In this study, Caco-2 cells were used to study the adhesion of bacteria to intestinal epithelial cells. These Caco-2 cells can undergo spontaneous differentiation and achieve the characteristics of mature enterocytes, such as structural polarization, tight junctions, apical microvilli and enzyme expression (Chantret *et al.*, 1988). Caco-2 cells have been widely used to study interactions of different bacteria (Wells *et al.*, 1999; Lievin-Le Moal *et al.*, 2002), including ETEC (Darfeuille-Michaud *et al.*, 1990; Gaastra and Svennerholm, 1996; Roselli *et al.*, 2003) with host epithelial cells.
In the present study, adhesion inhibiting activity after the major processing stages (soaking, cooking and fermentation) of the tempe manufacture will be investigated, in order to verify whether the fermentation step is required to generate its bio-activity. In addition, the effect on the ETEC K88 adhesion to human intestinal epithelial cells (Caco-2) will be measured, offering an independent alternative to piglet brush border cells.

Materials and methods

Soya bean products
Dry-dehulled full-fat yellow-seeded soya beans (*Glycine max*) were soaked overnight in tap water at 30ºC. In order to achieve an accelerated lactic acid fermentation during this soaking step, the soak water had been inoculated with naturally acidified soaking water ("backslop") (Nout *et al.*, 1987). Next, the soya beans were rinsed with tap water and cooked in fresh tap water for 20 min at a bean : water ratio of 1 : 3. Subsequently, the beans were cooled and their surface dried at room temperature, and were spread out on mesh trays for about 1 h. For the fungal inoculation of the soya beans a sporangiospore suspension was used, prepared by scraping off the sporangia from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU 573) grown on malt extract agar (CM59; Oxoid, Basingstoke, UK) for 7 days at 30ºC, and suspending them in sterile distilled water with 0.85% (w/v) NaCl and 0.1% (w/v) peptone. After inoculation with the sporangiospore suspension (10 ml kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans), the beans (batches of 450 g) were packed into hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated for 48, 72, 96 and 120 h. Fermented soya beans, as well as raw, soaked and cooked soya beans, were freeze-dried and ground (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) passing through a 0.5 mm sieve and were stored at -20ºC until further processing.

Freeze-dried products (20 g) were suspended and stirred for 1 h at room temperature in 200 ml of 35 mmol l^{-1} sodium phosphate buffer (pH 8.0), while the pH was adjusted with 2 mol l^{-1} NaOH every 30 min. The soluble extract was obtained by centrifugation (30 min, 25000 *g*, 20ºC), followed by filtration of the supernatant through a folded paper filter (no 311651, Schleicher & Schuell GmbH, Dassel, Germany), a second centrifugation step (30 min, 25000 *g*, 20ºC), and freeze-drying of the supernatants. These were stored at -20ºC and used as soya bean extracts in the experiments. Figure 1 outlines the preparation of the soya bean products studied.

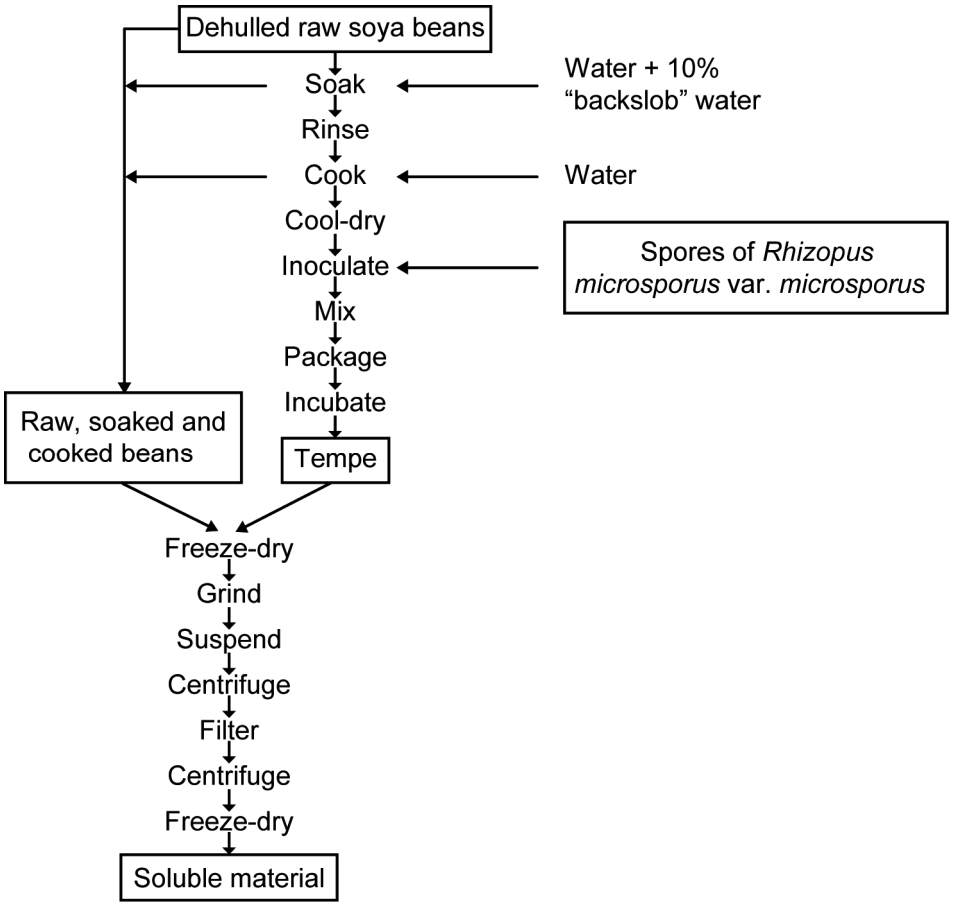

Figure 1: Flow diagram of the preparation of soya bean products and their extracts.

ETEC growth in the presence of soya bean extracts

Growth of ETEC in media containing 3 concentrations of soya bean extracts was monitored by measurement of optical density. Enterotoxigenic *Escherichia coli* (O149:K91:K88^{ac}) strain ID1000 (ETEC K88; source: Animal Science Group, Lelystad, The Netherlands) was cultured overnight in brain heart infusion (BHI) broth (237500, Becton Dickinson, France) at 37ºC. Cultures were centrifuged and re-suspended in BHI to a concentration of 10^6 CFU ml^{-1}. Soya bean extracts were dissolved at dry matter concentrations of 1, 2.5 or 10 g l^{-1} in BHI and 200 µl of these extracts were added to triplicate flat bottom wells of a sterile 96-well microplate (655161, Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands). Bacterial suspensions (50 µl) were added to the plate followed by incubation of the plate for 6 h at 37°C in a microplate reader. The optical density was monitored at a wavelength of 600 nm at intervals of 5 min with 5 s of shaking prior to each measurement (SpectraMax Plus 384; Molecular Devices Ltd, Wokingham, UK).

Adhesion of ETEC to brush border cells

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

The ETEC K88-positive (ID 1000) and K88-negative strain (ID1084) were grown overnight at 37ºC in BHI. The cultures were centrifuged (3000 *g*, 10 min) and washed twice with phosphate-buffered saline (PBS) (NaCl 136.89 mmol l^{-1}, KCl 2.68 mmol l^{-1}, Na_2HPO_4 8.1 mmol l^{-1}, KH_2PO_4 2.79 mmol l^{-1}, pH 7.2), followed by suspending the pellets in PBS, to a optical density of 0.75 correlating with 10^9 CFU ml^{-1}.

Freeze-dried soya bean extracts (10 mg) were dissolved in 1 ml PBS, mixed in a head-over-tail rotator for 1 h and centrifuged (10,000 *g*, 10 min) and further diluted to 2.5 and 1 g l^{-1}. Diluted extracts (50 µl) were mixed with 50 µl of ETEC K88 suspension and 50 µl of brush border cell suspensions, and the mixture was incubated at room temperature with continuous gentle shaking (100 min^{-1}) in a plate shaker for 1 h. The number of bacterial cells adhering to 12 individual brush border cells, was determined by phase contrast microscopy (magnification 1000x). The proportion of adhesion was

calculated as the average number of ETEC K88 per brush border cell, relatively to the adhesion with a PBS control.

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

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

Treatment No.[1]	Sample mixture[2]		Washing[3]	Addition (after 30 min)[4]	Adhesion (%)[5]
1 (pos. control)	BB	ETEC +	None	PBS	100 ± 2.7[a]
2 (neg. control)	BB	ETEC -	None	PBS	3.4 ± 1.6[b]
3	BB	ETEC +	None	TE	87.9 ± 4.4[a]
4	BB	TE	None	ETEC +	4.1 ± 2.1[b]
5	BB	TE	PBS (once)	ETEC +	61.6 ± 6.7[c]
6	ETEC +	TE	None	BB	7.4 ± 2.3[b]
7	ETEC +	TE	PBS (once)	BB	2.7 ± 1.9[b]
8	ETEC +	TE	PBS (twice)	BB	2.0 ± 1.1[b]
9	ETEC +	PBS	PBS (twice)	BB	72.3 ± 2.3[c]

[1] Treatment number corresponds to the sample mixture mentioned in columns 2 and 3.

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

[3] Washing by centrifugation of the sample mixture followed by suspension in PBS.

[4] Addition of the third component.

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

Adhesion of ETEC to Caco-2 epithelial cells

The human intestinal Caco-2 cell line (American Type Culture Collection, Rockville, MD, USA) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with addition of 25 mmol l^{-1} Hepes and 4.5 g l^{-1} glucose supplemented with 10% heat inactivated (30 min 56ºC) fetal bovine serum, 1% nonessential amino acids,

L-glutamine (2 mmol l^{-1}) and gentamycin (50 µg ml^{-1}) in an atmosphere of 5% CO_2/95% air at 37ºC. All cell culture reagents were obtained from Invitrogen (Breda, The Netherlands). Cells were sub-cultured after they had reached 70-90% confluence in plastic tissue culture flasks with a growth area of 75 cm^2 (Corning B.V, Schiphol-Rijk, The Netherlands). For bacterial adhesion experiments, cells were seeded in 12-well tissue culture plates (Corning B.V, Schiphol-Rijk, The Netherlands) at a concentration of $5x10^5$ cells ml^{-1} culture medium. In each well, 1 ml of the cell suspension was pipetted and the plates were left in the incubator for 3 weeks to allow differentiation. The medium was refreshed 3 times per week. Cells at passages 35-50 were used for experiments.

Freeze-dried soya bean extracts (12.5 mg) were suspended in 5 ml tissue culture medium (TCM) without serum and gentamicin. Extracts were centrifuged (3000 *g*, 10 min) and filtered through 0.22 µm filters (FP30/0.2CA-S, Schleicher & Schuell GmbH, Dassel, Germany). Cytotoxicity of tempe extracts to Caco-2 cells was tested using the lactate dehydrogenase (LDH) leakage test (Mitchell *et al.*, 1980).

The ETEC K88 strain was cultured overnight in BHI at 37ºC. The culture was washed twice and re-suspended in TCM, to a density of about 10^9 CFU ml^{-1}. Caco-2 cells were washed once with PBS (pH 7.4) and 1 ml of soya bean extract was added to triplicate wells, followed by 50 µl of bacterial suspension. To allow ETEC adhesion, the plates were incubated for 1 h at 37ºC. Nonadhered bacteria were removed by three washes with 1 ml of PBS. Caco-2 cells were then lysed with 1 ml 1% triton-X-100 (T8787, Sigma) and viable ETEC K88 were quantified by plating appropriate serial dilutions of lysates on Tryptone Soy agar (TSA) (CM 0131, Oxoid, Basingstoke, UK). To quantify bacterial adhesion to Caco-2 cells, each combination of soya bean extract and Caco-2 cells was tested in three independent replications performed on different days and in triplicate tissue culture wells. The adhesion was plotted relatively to a control, without soya bean extract, which was defined as 100%.

ETEC adhesion to soya bean extracts

Bacterial adhesion to soya bean extracts was measured according to Becker *et al.* (2007). The freeze-dried soya bean extracts were suspended in PBS (pH 7.2) to concentrations of 1, 2.5 and 10 g l^{-1} and centrifuged (460 *g*, 5 min, 21ºC). The flat-bottomed wells of high-binding polystyrene microtitration plates (Microlon F plate 655092; Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) were coated with 300 µl of the supernatant and incubated overnight at 4°C. Non-coated wells were included as negative controls in each plate. Subsequently, plates were washed with 300

µl PBS buffer to remove nonbound material, and the plates were blocked by adding 300 µl of 10 g l^{-1} Bovine Serum Albumin (BSA) A7906; Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS per well, and incubation at 4°C for 1 h. Subsequently, the plates were washed twice with 300 µl of PBS to remove excess BSA. ETEC was grown overnight, washed and suspended in PBS to an optical density of 0.02 (600 nm, path length 1 cm) corresponding to about 10^7 CFU ml^{-1}, and 300 µl of this bacteria suspension was added into the wells. Bacteria were allowed to adhere to the coated soya bean extracts at room temperature for 30 min. Then, the wells were washed three times with 300 µl of PBS to remove nonadhered bacteria, and 250 µl BHI broth was added to each well. The microtitre plate was placed in a microplate reader (SpectraMax plus 384; Molecular Devices Ltd, Wokingham, UK), where it was incubated at 37°C and shaken at medium intensity for 5 s prior to every reading. The OD was determined at a wavelength of 600 nm in intervals of 5 min. All readings were performed in triplicate and the experiment was replicated once. The optical density measurement was used as a tool to compare numbers of adhered bacteria to the coated soya bean extracts. A correlation between the time-dependent detection of the growth by optical density measurement and the initial cell density of adhered bacteria in plates is demonstrated in Becker *et al.* (2007). The OD was determined at a wavelength of 600 nm in intervals of 5 min. All readings were performed in triplicate and the experiment was replicated.

Statistical analyses

Significance of adhesion of ETEC to Caco-2 cells was determined by the Student's *t*-test assuming equal variances. The significance of the other experiments was evaluated by comparing means using one or two-way ANOVA, followed by the Bonferonni posttest. Results were expressed as mean ± SEM and differences were considered significant when $P < 0.05$. Statistics were performed with Graphpad Prism version 4.03 for Windows (Graphpad Software, San Diego, CA, USA).

Results

Effect of soya bean extracts on the growth of ETEC

Figure 2 shows the effect of raw, soaked, cooked and fermented soya bean extracts added to BHI in three concentrations, on the growth of ETEC K88. Optical densities of cultures, growing in soya bean extract medium, after 4 h incubation (mid-logarithmic phase) were compared with the control culture in BHI. None of the extracts inhibited

the growth of ETEC K88. Instead, almost all fermented and nonfermented soya bean extracts showed a small but significant increase in optical density compared with the control. These results showed no antibacterial effect of the soya bean products to ETEC K88 within the tested concentrations. There were also no significant concentration effects of the soya bean extracts within a group of soya bean extracts on the growth of ETEC K88.

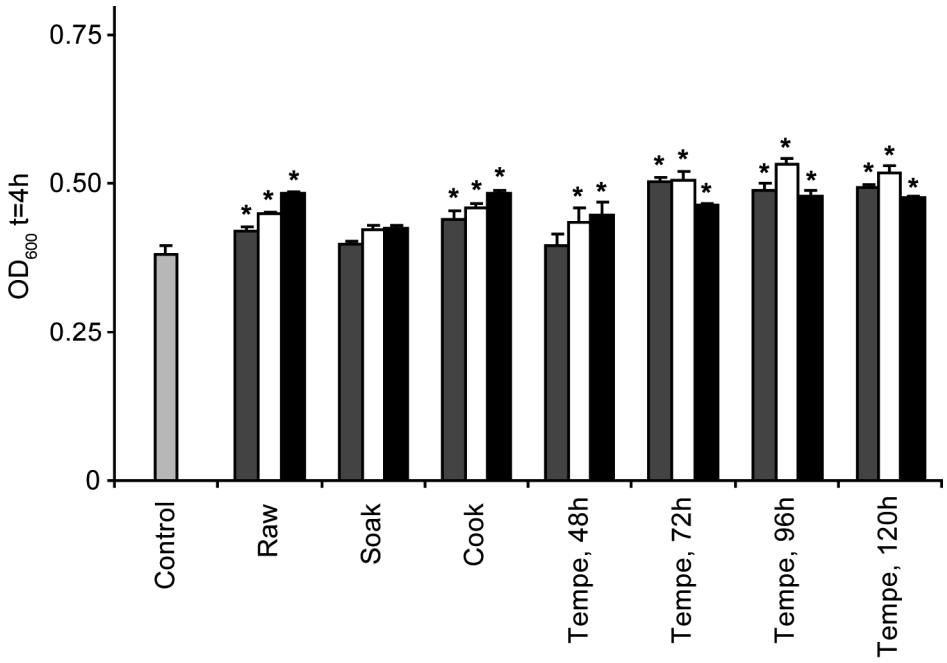

Figure 2: Effect of soya bean extracts on the optical density of ETEC K88 in BHI. Figure shows OD_{600} after 4 h of growth of ETEC K88 in BHI (control: light grey bar) and BHI with added soya bean extracts 1 g l^{-1} (grey bar), 2.5 g l^{-1} (white bar) and 10 g l^{-1} (black bar) concentrations. Bars with asterisk (*) are significantly different from control. Error bars represent SEM of three independent experiments.

Adhesion of ETEC K88 to brush border cells

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

In the presence of 10 g l^{-1} soya bean extracts, a complete inhibition of the adhesion was observed with all extracts. At the level of 2.5 g l^{-1}, raw, soaked, and cooked soya bean extracts resulted in almost 50% inhibition of adhesion compared with the control, whereas the tempe extracts reduced adhesion to levels similar to the negative control.

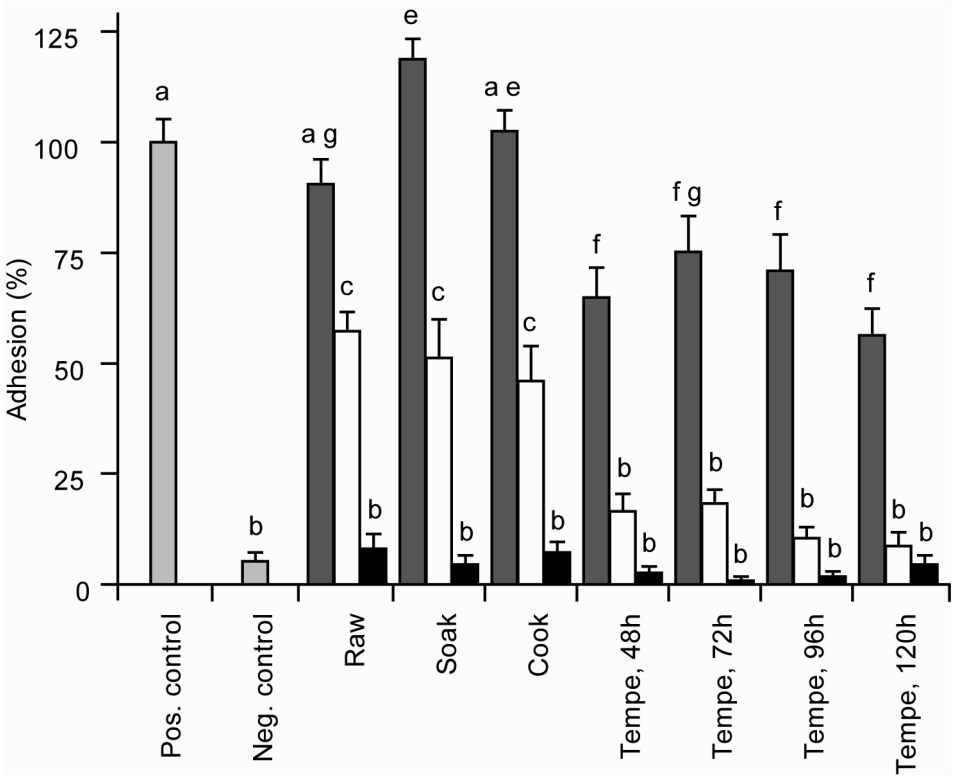

Figure 3: Adhesion of ETEC K88 to piglet intestinal brush border cells. Adhesion is expressed relative to a control (100%) without added soya bean extract (light grey bar). Soya bean extracts were added as 1 g l^{-1} (grey bar), 2.5 g l^{-1} (white bar) and 10 g l^{-1} (black bar) concentrations. The negative control represents adhesion of an ETEC-negative nonadhering strain. Error bars represent the SEM of 12 brush border cell counts. Means without a common letter differ significantly.

At the level of 1 g l^{-1}, there was only a significant inhibition of ETEC adhesion observed with the fermented soya bean (tempe) extracts. Longer fermentation times (48-120 h) did not significantly affect the inhibition activity.

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

Adhesion of ETEC K88 to Caco-2 epithelial cells

In the absence of soya bean extracts, approximately 2 x 10^6 CFU ml^{-1} of ETEC cells adhered to Caco-2 cells; this value was defined as 100% adhesion. In figure 4, the effect of 2.5 g l^{-1} of soya bean extracts on the adhesion of ETEC K88 to Caco-2 cells is shown.

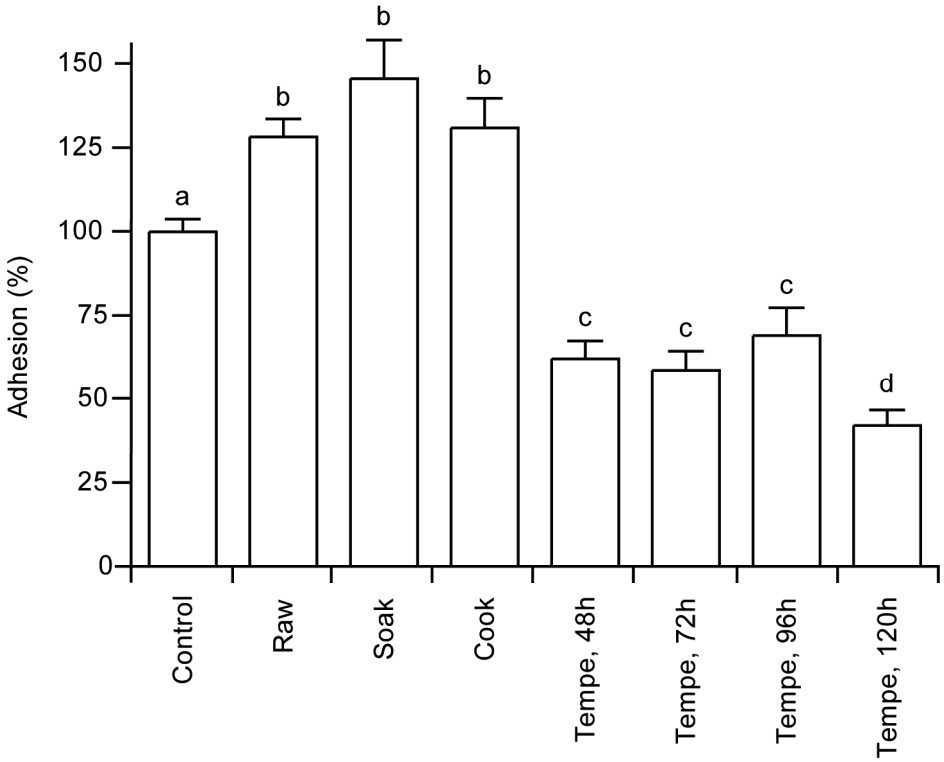

Figure 4: Adhesion of ETEC K88 to Caco-2 cells, as influenced by soya bean extracts. Bacterial adhesion is expressed relative to a control (100%) without added soya bean extract. Soya bean extracts were added at 2.5 g l^{-1}. Error bars represent SEM values of three independent experiments carried out in triplicate (n = 9). Means with a different letter differ significantly.

We estimated that this soya concentration is of an order of magnitude that can be encountered in the intestine after consumption of a serving of tempe. The used concentration (2.5 g l^{-1}) did not cause any damage to the Caco-2 cells themselves as tested with a LDH-leakage test (data not shown).
Extracts of raw, soaked and cooked soya beans showed a slight but significant increase of ETEC K88 adhesion, compared with the control. Treatment with the tempe extracts resulted in a significant inhibition of adhesion of almost 50% compared with the control. Longer fermentation times did not show additional effect on the inhibitory activity on the adhesion, only 120-h fermented tempe showed an even lower adherence percentage.

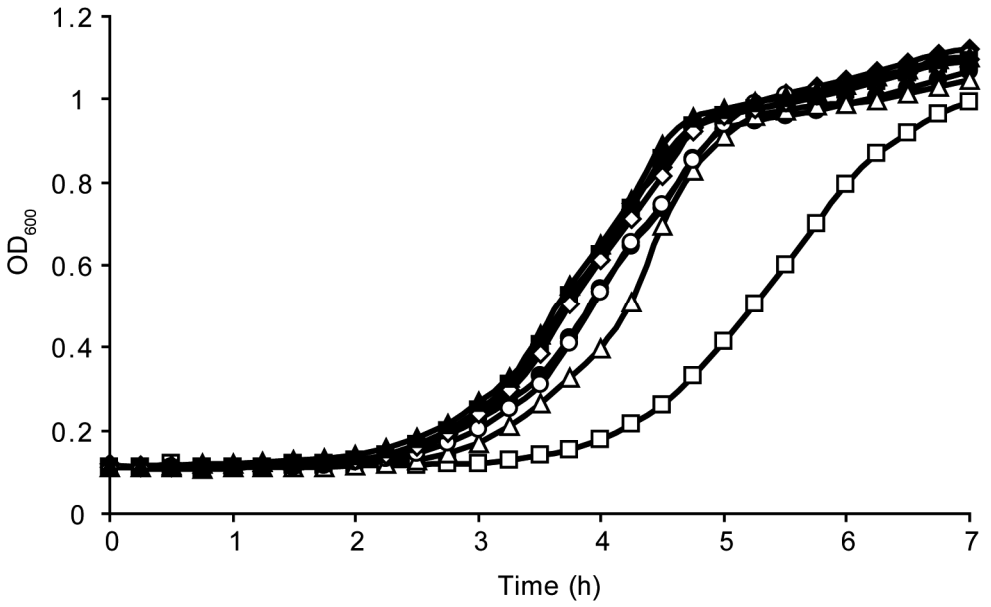

Figure 5: Growth curves of adhered ETEC K88 to soya bean extracts. Figure shows the growth of adhered bacteria on plates coated with 2.5 g l^{-1} of soya bean extract of one replication in triplicate. Lines with different symbols represent the control (open square), raw (closed circle), soaked (closed diamond) and cooked (open triangle) soya beans and soya beans fermented for 48 h (open circle), 72 h (closed square), 96 h (open diamond) and 120 h (closed triangle). Optical density was measured every five min, but for clarity only 15 min time point are shown in this figure. Error bars of the three measurements are not visible in the figure since they were smaller than the symbols shown.

ETEC K88 adhesion to soya bean extracts

Becker *et al.* (2007) demonstrated a correlation between the time-dependent detection of the growth by optical density measurement, and the initial cell density in plates. Higher initial numbers, for instance of adhering cells, result in shorter detection times of bacterial growth upon incubation. Figure 5 shows the optical density curves of ETEC K88 adhered to a plate coated with 2.5 g l^{-1} soya bean extract as a representative of the curves obtained. Based on this correlation and the observations in figure 5, significantly more ETEC K88 was bound to all of the soya bean extracts, compared with the control.

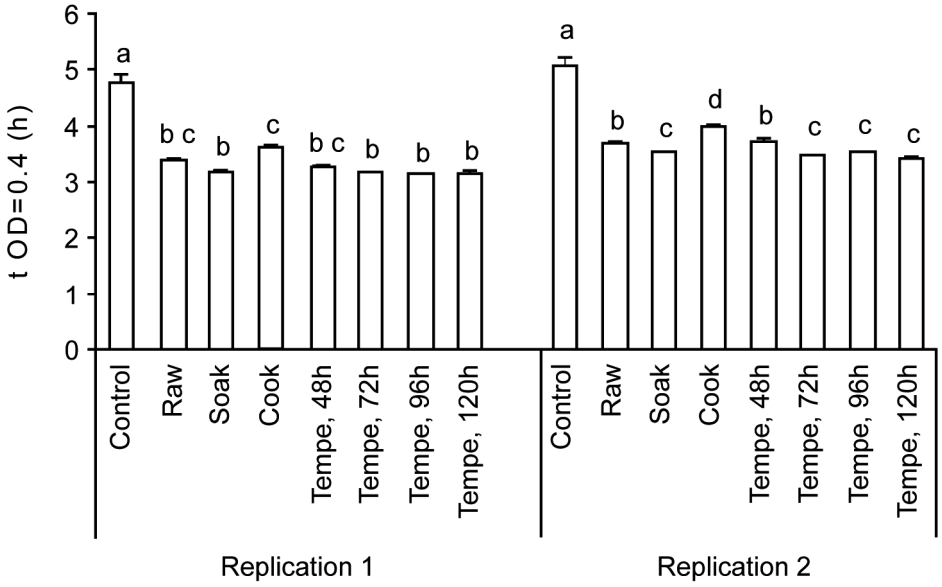

Figure 6: Adhesion of ETEC K88 to soya bean extracts. Bacterial adhesion is expressed as the mean time (h) to reach OD = 0.4 at 600 nm. For clarity only results of 2.5 g l^{-1} soya bean extracts are presented. Error bars represent SEM of three measurements of the extracts and nine measurements of the control. Left side of the figure shows replication 1 and the right side replication 2. Means without a common letter differ significantly.

Figure 6 shows two independent replications of optical density (OD) curves for 2.5 g l^{-1} soya bean extract concentration, compared at OD = 0.4 level. This level was selected as a criterion for its location in the linear part of all the optical density curves (see figure 5) and it corresponds with about 10^8 CFU $ml^{-1.}$. A comparison of the mean times to achieve OD = 0.4 reveals the same trend in both the replications, although the

significant ($p < 0.05$) difference of about 20 min occurred due to variations in initial bacterial load (namely 2.5×10^7 CFU ml^{-1} for replication 1 and 1.9×10^7 CFU ml^{-1} for replication 2) and timing of the experiments.
The wells coated with the extracts of tempe fermented for 72, 96 or 120 h all showed similar growth curves in both replications, reaching a mean OD = 0.4 after 3.2 ± 0.01 h (mean ± SEM) compared with the control 4.8 ± 0.13 h (mean ± SEM) for replication 1. Tempe extracts gave similar adhesion as the soaked bean extracts. The adherence of ETEC to raw and cooked soya bean extracts is significantly lower than to tempe extracts.
Other soya bean concentrations (1 and 10 g l^{-1}) did not show significant different results and are not shown.

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

Discussion

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

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

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

The intrinsic microorganisms (i.e. originating from intestinal microbiota) had been removed during the preparation and purification of the brush border cells as was confirmed by their absence in microscopical observation. Consequently, possibilities of interference of intrinsic microbiota with ETEC were not included in this study. However, *in vivo* studies (Kiers *et al.*, 2003) have shown that anti-diarrhoeal effects are significant *in vivo*, implying that intrinsic microbiota has no nullifying effect. Inhibition of ETEC K88 adhesion to brush border cells was reported previously for several tempe extracts (Kiers *et al.*, 2002). In the present study, all processing stages of the tempe process were considered for their inhibitory effect on adhesion to brush border cells. Although not as effective as tempe, the extracts of raw, soaked, and cooked soya beans also inhibited ETEC K88 adhesion, and for all soya bean extracts, higher inhibitory activity was observed at higher extract concentrations. The extract concentrations tested (1, 2.5 and 10 g l^{-1}) were physiologically relevant, considering a serving size of 100 g of tempe of which at least 30% of dry matter is solubilised by digestion; in the intestinal tract this would be diluted to concentrations of at least 10 g l^{-1} (Kiers *et al.*, 2000). We also confirmed that this concentration did not cause any artefacts in our experiments by undue damages to the Caco-2, or brush border cells.

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

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

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

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

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

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

Acknowledgements

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Chapter 3

Soya bean tempe extracts show antibacterial activity against *Bacillus cereus* cells and spores

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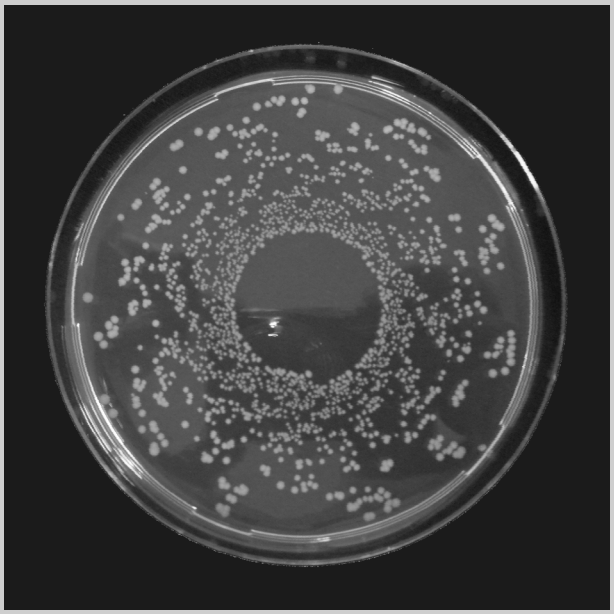

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Abstract

Aims: Tempe, a *Rhizopus* ssp. fermented soya bean food product, was investigated for bacteriostatic and/or bactericidal effects against cells and spores of the food-borne pathogen *Bacillus cereus*.

Methods and results: Tempe extract showed a high antibacterial activity against *Bacillus cereus* ATCC 14579 based on optical density and viable count measurements. This growth inhibition was manifested by a 4 log CFU ml^{-1} reduction, within the first 15 min of exposure. Tempe extracts also rapidly inactivated *Bacillus cereus* spores upon germination. Viability and membrane permeability assessments using fluorescence probes showed rapid inactivation and permeabilization of the cytoplasmic membrane confirming the bactericidal mode of action. Cooked beans and *Rhizopus* grown on different media did not show antibacterial activity, indicating the unique association of the antibacterial activity with tempe. Subsequent characterization of the antibacterial activity revealed that heat treatment and protease addition nullified the bactericidal effect, indicating the proteinaceous nature of the bioactive compound.

Conclusions: During fermentation of soya beans with *Rhizopus,* compounds are released with extensive antibacterial activity against *B. cereus* cells and spores.

Significance and Impact of Study: The results show the potential of producing natural antibacterial compounds that could be used as ingredients in food preservation and pathogen control.

Introduction

Tempe is the collective name for a sliceable mass of pre-cooked fungal fermented beans, cereals or some other food processing by-products bound together by the mycelium of a living mould (mostly *Rhizopus* spp.). Yellow-seeded soya beans are the most common and preferred raw material to make tempe. Traditional manufacture of tempe includes two major steps, namely a soaking process of the raw beans, where beans are acidified by natural occurring lactic acid bacteria. This is followed by a fungal fermentation initiated by an added mould starter culture. The purpose of the fermentation is not as much to enhance preservation, but rather the modification of ingredients and an increase of the nutritional properties because of enzymatic activity (Nout and Kiers, 2005).

Previous investigations by Wang *et al.* (1969) showed that tempe contains antibacterial properties against some Gram-positive bacteria. These were associated with the mould *Rhizopus oligosporus*. In 1992, an antibiotic substance produced by *Rhizopus oligosporus* grown in culture broth was purified (Kobayasi *et al.*, 1992) and found to be active against *Bacillus subtilis* vegetative cells. Neither further investigations of this antibacterial effect, nor the effect of tempe extracts on *Bacillus* spores have been published since.

The present study deals with the antibacterial effect of soya bean tempe on *B. cereus* vegetative cells and spores. *Bacillus cereus* is a Gram-positive, spore forming bacterium, able to cause two types of food poisoning: the diarrhoeal type by enterotoxin production in the small intestine and the emetic type by cereulide, a toxin produced during growth in food (Kotiranta *et al.*, 2000). *Bacillus cereus* is ubiquitous in nature and frequently isolated from soil and plants. As a common inhabitant of soils, the organism can easily be transmitted to vegetation and hence to foods (Notermans and Batt, 1998; Stenfors Arnesen *et al.*, 2008).

The potential use of natural antimicrobials such as bacteriocins, for the improvement of microbial quality and safety of food has stimulated intensive research efforts in recent years (Galvez *et al.*, 2007; Settanni and Corsetti, 2008). An antibacterial compound produced by tempe processing may be of considerable interest, because of its natural food-based origin. Inhibition of spoilage caused by outgrowth of bacterial spores may be an additional target for application, because of the resistance of spores to several preservation techniques.

Pathogenic organisms are also developing more resistance to conventional antibiotics. This problematic trend has generated an increased interest in the pharmacological

application of antimicrobial peptides to cure infections (Epand and Vogel, 1999). Food-based antibacterial compounds may not only be important for food preservation but can possibly also be used to control pathogens and prevent human infections (Cotter *et al.*, 2005).
In the present study the antibacterial activity of tempe is investigated with *B. cereus* as the target organism. The activity is not only tested against vegetative cells, but also against spores. Furthermore, the origin of the active component by testing the intermediate stages of processing and fermentation strains separately is determined. Experiments are performed to give details about the mode of action and characteristics of the active component.

Materials and methods

Tempe processing

Dry-dehulled full-fat yellow-seeded soya beans (*Glycine max*) were soaked overnight in tap water at 30ºC. Tap water was enriched with 10% naturally acidified soaking water from previous soya bean soaking steps, according to the "backslop" procedure (Nout *et al.*, 1987). After overnight incubation soya beans were rinsed with tap water and cooked in fresh tap water for 20 min (ratio soaked beans : water 1 : 3) and cooled by evaporation of adhering moisture at room temperature. Soya beans were inoculated with a sporangiospore suspension of *Rhizopus microsporus* var. *microsporus* (LU 573). This suspension was prepared by scraping off the sporangia from pure cultures grown on malt extract agar slants (CM59; Oxoid, Basingstoke, UK) for 7 days at 30ºC, in peptone physiological salt (PPS) (0.85% NaCl and 0.1% peptone solution). After inoculation with the sporangiospore suspension (10 ml kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans), the soya beans (batches of 450 g) were packed in hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated at 30ºC for 24, 48, 72, 96 and 120 h.

Soya extract preparation

Fermented soya beans (tempe) and cooked soya beans, were freeze-dried and ground passing through a 0.5 mm sieve (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) and were stored at -20ºC until further processing.
Freeze-dried products were suspended in distilled water (60 g l^{-1}) and stirred with a magnetic stirrer for 3 h at room temperature. The pH was continually checked and adjusted to pH 8.0 with 1 mol l^{-1} NaOH. To obtain clear supernatants, the soluble

extract was obtained by three consecutive centrifugation steps (10 min, 10000 *g*, 20ºC). Supernatants were freeze-dried and soluble dry matter was stored at -20ºC and used as soya bean soluble dry matter in experiments.

Mould extract preparation

Rhizopus microsporus var. *microsporus* (LU 573) was grown on different liquid and solid media, to test the antibacterial activity of the mould biomass. *Rhizopus* sporangiospores were inoculated in malt extract broth (MEB, CM59; Oxoid, Basingstoke, UK) and mineral medium (MIB) containing NH_4Cl (5 mmol l^{-1}), D-glucose (25 mmol l^{-1}), $MgSO_4.7H_2O$ (1.3 mmol l^{-1}), $ZnSO_4.7H_2O$ (0.3 mmol l^{-1}), $MnSO_4.4H_2O$ (0.2 mmol l^{-1}), $FeCl_3.6H_2O$ (70 µmol l^{-1}), $CuSO_4.5H_2O$ (40 µmol l^{-1}) and EDTA (1.2 mmol l^{-1}) in a 0.01 mol l^{-1} K-phosphate buffer and grown for 4 days in a shaking incubator (30°C, 200 rev min^{-1}). Mycelium was collected on a folded paper filter (no. 311651; Schleicher & Schuell GmbH, Germany) and washed three times with PPS by centrifugation (10 min, 3000 *g*, 20°C). The pellet was freeze-dried and stored at -20°C until use. After removal of the mould biomass, the post-growth media were also freeze-dried and prepared for the activity assay, to test the effect of released fungal components. *Rhizopus. microsporus* was also grown on plates of Malt Extract agar (MEA) and cooked soya bean agar (CSBA) (30 g l^{-1} freeze-dried cooked soya beans powder and 15 g l^{-1} agar) for 4 days at 30°C, followed by scraping off the mycelium from the agar plates and freeze drying of the mycelium. The collected mycelium extract and postgrowth media extracts were prepared in the same way as described for the soya bean extracts.

Bacteria and culture conditions

B. cereus strain ATCC 14579 was used for investigation of the tempe antibacterial effect. Stock cultures were stored at -80ºC in 25% glycerol. Prior to experiments, strains were inoculated in Brain Heart Infusion broth (BHI, 237500, Becton Dickinson, France) and grown for 16-18 h at 30ºC in a shaking incubator (200 rev min^{-1}).

Spores were obtained from an overnight culture of *B. cereus* (ATCC 14579) grown in tubes with 5 ml Luria Broth (LB; 241420, Becton Dickinson, France) at 30°C and 200 rev min^{-1} rotary shaking. Then cells were inoculated in maltose sporulation medium (MSM) based on the sporulation medium used by Schaeffer *et al.* (1965) fortified with sporulation elements of the defined medium for *B. cereus* and maltose to increase the yield. The MSM contained Nutrient Broth (8 g l^{-1}; NB, 234000, Becton Dickinson, France), maltose (10 mmol l^{-1}), $(NH_4)_2SO_4$ (5 mmol l^{-1}), $MgCl_2$ (1 mmol l^{-1}), $Ca(NO_3)_2$

(1 mmol l^{-1}), $FeSO_4$ (1 mmol l^{-1}), $MnSO_4$ (66 µmol l^{-1}), $ZnCl_2$ (12.5 µmol l^{-1}), $CuCl_2$ (2.5 µmol l^{-1}), Na_2MoO_4 (2.5 µmol l^{-1}) and $CoCl_2$ (2.5 µmol l^{-1}). Sporulation was performed in 50 ml MSM in 250-ml Erlenmeyer flasks, at 30°C and 200 rev min^{-1} rotary shaking. Sporulation efficiency was determined by microscopical observation and droplet plating before and after heating of sporulating cultures. In order to obtain spore batches containing (>95%) spores only, as controlled microscopically, spores were washed in 10 rev min^{-1} potassium phosphate buffer (pH 7.4), at least 10 times during the first 3 weeks, before starting any further experiments. Spore suspensions were stored at 4ºC and washed weekly to prevent spontaneous germination.
The antibacterial spectrum of tempe was tested on the following strains: *B. cereus* ATCC 10987, NIZO B437, PAL 20, PAL 28, 55, *Bacillus weihenstephanensis* DSM11821T and *Bacillus subtilis* B20010. All strains were inoculated in BHI broth and grown for 16-18 h at 30ºC with rotary shaking (200 rev min^{-1}) before experiments.

Antibacterial activity assay
Two different methods were used: (a) monitoring bacterial biomass by optical density and (b) viable count enumeration. In both methods, soya bean dry matter (20 g l^{-1}) was dissolved in distilled water by mixing for 3 h on a head-over-tail rotator, followed by centrifugation (10 min, 16000 *g*, 20°C) and filter sterilization (0.22 µm, FP30/0.2CA-S, Schleicher & Schuell GmbH, Dassel,Germany). The pH of the extract was around 7. The sterile filtrate was diluted 1 : 1 in double concentrated BHI to achieve a 10 g l^{-1} extract in growth medium or was diluted further in BHI.
For optical density (OD) measurement, 50 µl of a suspension containing 10^6 CFU ml^{-1} vegetative cells or spores were inoculated in 200 µl of BHI with or without tempe extract, in 96-well microtiter plates in triplicate. For each experiment, inoculation levels were verified by viable counts on BHI-agar. Microtiter plates were incubated in a spectrophotometer plate reader (SpectraMax Plus 384, Molecular Devices Ltd, Workingham, Berkshire, UK) at 30ºC up to 24 h. Optical density was measured every minute at 600 nm. Prior to each measurement the plate was shaken for 45 s to ensure homogeneity and optimal aerobic growth conditions. The antibacterial activity is expressed as the growth delay in h:

$$\text{Growth delay (h)} = t_{OD=0.6,\,\text{exp}} - t_{OD=0.6,\,\text{control}}$$

Growth delay is defined as the time *B. cereus* growing in BHI with tempe extract (exp) needed to reach an OD of 0.6 minus the time *B. cereus* in BHI (control) needed to

reach this OD-point. Data represent means and standard deviations of triplicate measurements.
During viable count enumeration experiments, BHI broth with or without tempe extract was inoculated in the same ratio medium/inoculation (4 : 1) as performed in the optical density measurement and was incubated at 30ºC with continuous shaking (200 rev min^{-1}). Samples were taken at different time points, diluted with PPS and plated in duplicate on BHI-agar.

Microscopic observations
During viable count enumeration, *B. cereus* cells and spores were investigated microscopically. Bacterial cells were collected by centrifugation (3 min, 1520 *g*, 20ºC), suspended in PPS and observed by phase-contrast microscope (magnification 1000x).
For viability determination, a fluorescence double staining technique was used. The green fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI) (Live/Dead *Bac*light Viability Kit, InVitrogen L7012; Breda, The Netherlands) were mixed in a 1 : 1 dye mixture. *B. cereus* cells or spores were re-suspended to 10^8 CFU ml^{-1} in a bovine serum albumin-saline solution (2.5 g l^{-1} BSA with 0.15 mol l^{-1} NaCl); 1 ml of cell suspension was mixed with 2 µl dye mixture and incubated in the dark on ice for 15 min. The stained *B. cereus* cells were observed with a fluorescence microscope.

Effect of pH, enzymes and heat on antibacterial activity
Tempe extracts were prepared as described for the antibacterial activity assays. Different treatments were performed as follows:
To investigate any inhibitory effect of pH, the extracts were adjusted by addition of 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl from pH 2.0 up to pH 9.0. For low pH (2.0, 3.0 and 4.0), the tempe extract solution was adjusted with 1 mol l^{-1} HCl, mixed for an hour, centrifuged and supernatant was neutralized again to pH 7 with NaOH before antibacterial activity was measured.
Tempe extracts were incubated with different enzymes of ample concentration (10 mg ml^{-1}) at 30ºC with continuous shaking (200 rpm) for an extended period of 4 h to allow degradation of all susceptible material. The following enzymes were used: Pronase E (P5147), Proteinase K (P8044), Trypsin (T0134), α-Chymotrypsin (C4129) and Protease type XII (P2143) all from Sigma-Aldrich. Enzymes were not inactivated, because *B. cereus* growth was not affected by their presence as confirmed by control experiments with enzyme solutions only (data not shown).

Tempe extracts were heated for 20 min at 20, 30, 40, 50, 60, 70, 80, 90 and 100°C in a water bath. After heating the samples were immediately cooled down till 4°C, followed by measurement of their antibacterial activity.
After the treatments mentioned previously, tempe extracts were centrifuged and diluted with BHI as performed in antibacterial activity assay. Then, they were inoculated with *B. cereus* and the antibacterial activity was measured by optical density growth measurements.

Results

The impact of soya bean extracts on *Bacillus cereus* vegetative cells growth
Growth of *B. cereus* was assessed in BHI with or without addition of soya bean extracts. Figure 1A shows the optical density increase during 24-h incubation at 30ºC and figure 1B shows the viable count enumeration of *B. cereus* using the same soya beans extracts performed simultaneously. The growth of *B. cereus* in BHI with or without addition of 10 g l^{-1} cooked beans was similar, whereas the growth of *B. cereus* in BHI with 10 g l^{-1} tempe added showed a growth delay of 12.31 h ± 0.22 (mean ± standard deviation) (figure 1A) and a reduction in viable count of 3.7 log CFU ml^{-1} (figure 1B). This reduction in viable count occurred within the first 30 min of measurement, indicating a remarkable bactericidal effect.
Bacillus cereus viability was also analyzed microscopically using fluorescent live/dead staining. After 15 min of incubation *B. cereus* cells in BHI with added tempe extract were fluorescent red-stained which means that the PI stain entered the cells indicative of membrane damage (data not shown).
After 24 h incubation of *B. cereus* in BHI with added tempe extract, lower numbers of viable cells were reached (figure 1B). Microscopic observations, however, showed that *B. cereus* formed long chains of rods in all three BHI with added tempe extracts samples compared to cooked and BHI without addition of tempe. This could result in less colonies on the agar plates and an underestimation of the viable cell count after 24 h. This aggregation of bacterial cells was not observed after 15 min.

The impact of soya bean extracts of the tempe processing stages on *Bacillus cereus* vegetative cells growth
During tempe processing, antibacterial activity of intermediate soya products was observed using the optical density assay. Growth delay caused by soya and tempe extracts of the successive stages of the processing are shown in figure 2A.

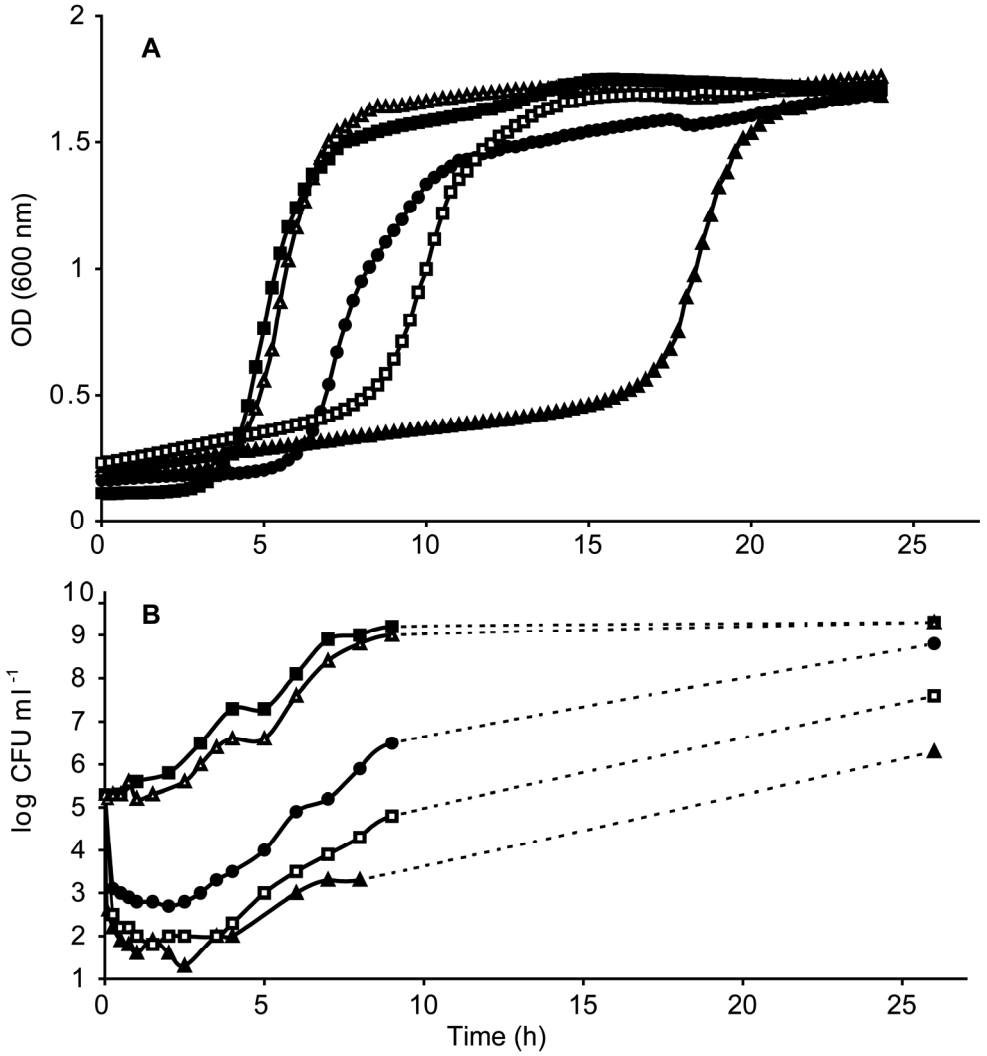

Figure 1: Influence of tempe extracts on vegetative cells of *B. cereus* 14579. (A) Growth of *B. cereus* 14579 by optical density in BHI (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square) and tempe 10 g l^{-1} fermented for 24 h (closed triangle). For clarity of the figure, data points at 15-min intervals are shown. (B) Viable count enumeration of *B. cereus* 14579 growth in BHI (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square) and tempe 10 g l^{-1} fermented for 24 h (closed triangle). The dotted lines are predictions of the growth between 9 and 26 h.

Growth delays caused by raw, soaked and cooked soya beans, *i.e.*, soya extracts of the processing steps before mould inoculation, were negligible. No effect of the soaking step, during which lactic acid bacteria actively develop, was observed. After inoculation with *R. microsporus* and incubation for 24 h, the growth delay of *B. cereus* had increased up to 9.36 ± 0.5 h (mean ± standard deviation). Longer fermentation times resulted in less growth delay decreasing to 5.33 ± 0.08 h (mean ± standard deviation) after 120 h of fermentation.

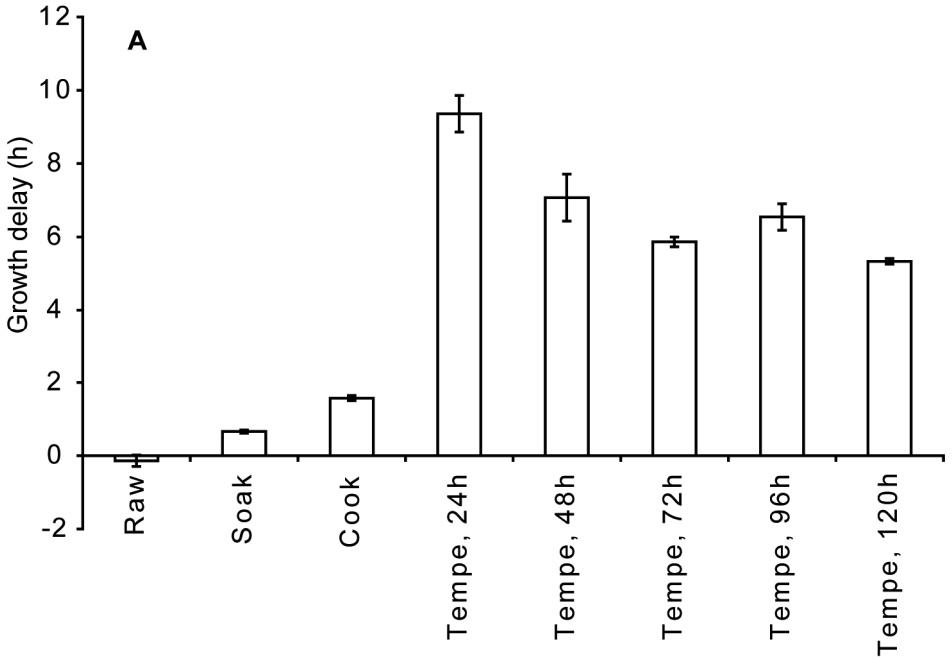

Figure 2A: Growth delay measured by optical density of *B cereus* 14579 after exposure to several extracts. (A): Growth delay of *B. cereus* 14579 after exposure to extracts (10 g l^{-1}) of several soya bean products during tempe processing. X-axis represents the several stages during tempe processing, respectively: raw soya beans (raw), soaked soya beans (soak), cooked soya beans (cook), tempe fermented for 24 h (tempe, 24 h), tempe fermented for 48 h (tempe, 48 h), tempe fermented for 72 h (tempe, 72 h), tempe fermented for 96 h (tempe, 96 h), tempe fermented for 120 h (tempe, 120 h).

The antibacterial activity of *R. microsporus* grown on different media and their postgrowth media was also tested (figure 2B). *Rhizopus microsporus* grown on the different media, MEB, MIB, MEA and CSBA showed a growth delay <1.8 h, whereas

the tested tempe extract showed a delay of 14.9 ± 2.36 h. Also the postgrowth media showed no activity towards *B. cereus*.

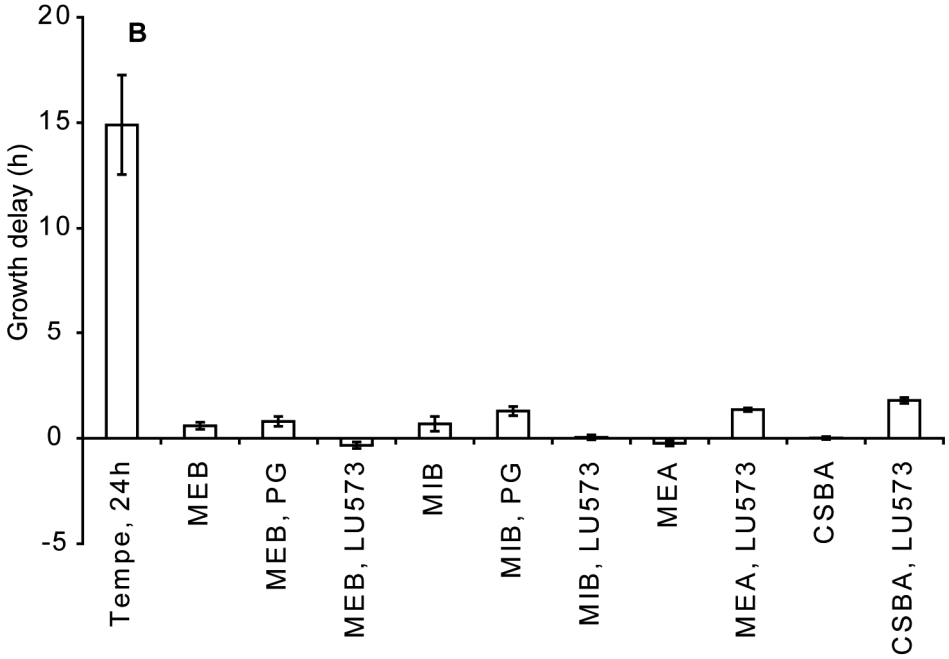

Figure 2B: Growth delay measured by optical density of *B cereus* 14579 after exposure to several extracts. (B): Growth delay measured of *B. cereus* after exposure to mould biomass grown in different media, and to corresponding postgrowth media. The concentration of the tempe (fermented for 24 h) extract is 10 g l^{-1} and of the mould extract 1 g l^{-1}. MEB, malt extract broth; MIB, Mineral medium; MEA, Malt extract agar; CSBA, Cooked soya bean agar; PG, Postgrowth medium; LU573, inoculated with *Rhizopus microsporus* LU 573.

Effect of soya bean extracts on germination and outgrowth of *B. cereus* spores

Bacillus. cereus spores (log 9 CFU ml^{-1}) were incubated with and without soya bean extracts and monitored for their germination and growth. Figure 3A presents the results of the OD measurement. All samples showed a similar germination, as was manifested by an OD drop within 1 h reflecting transition from the phase bright dormant phase into the phase dark germinated phase (60% loss of optical density represents a germination of 100%).

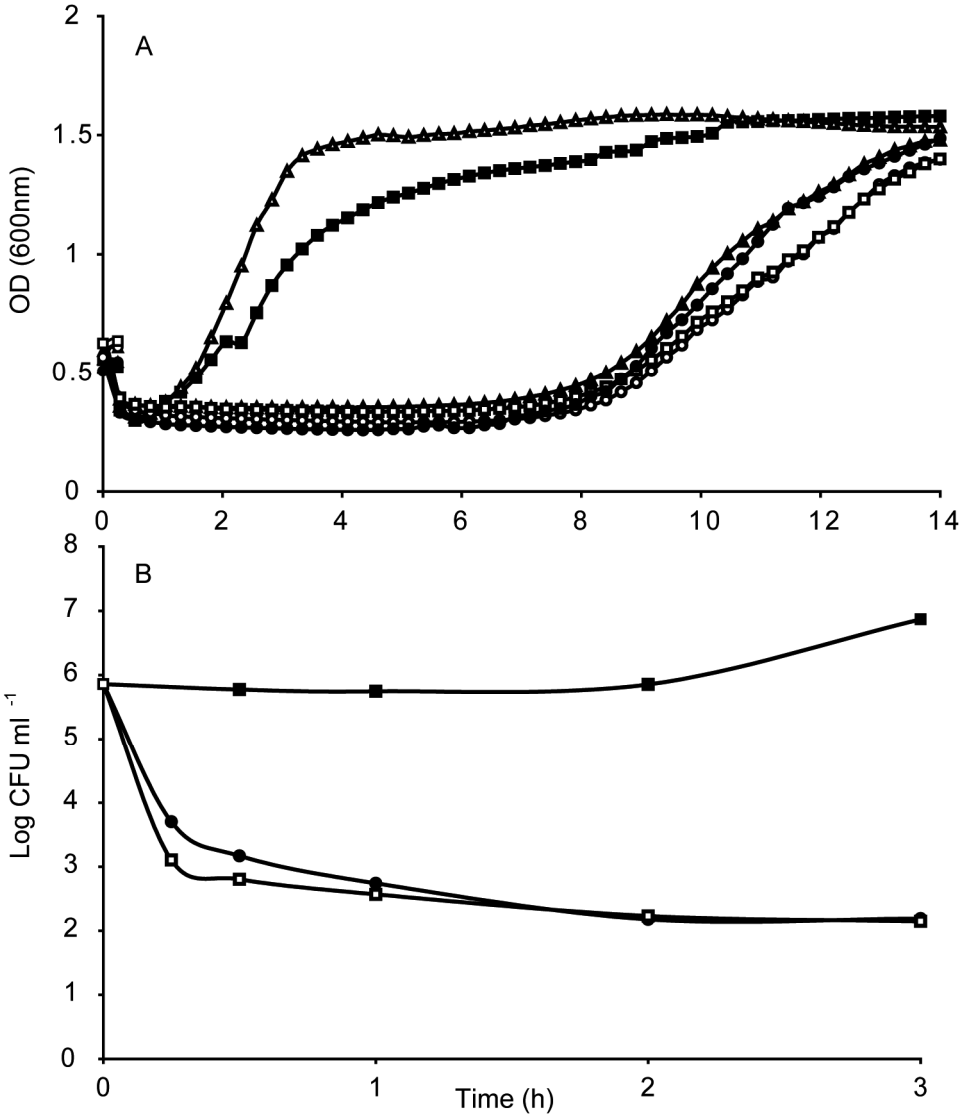

Figure 3: Influence of tempe extracts on spores of *B. cereus* 14579. (A) Germination and outgrowth of spores by optical density in Brain Heart Infusion (BHI) (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square), tempe 1 g l^{-1} fermented for 24 h (open circle) and tempe 10 g l^{-1} fermented for 24 h (closed triangle). For clarity of the figure, data points at 15-min intervals are shown. (B) Viable count enumeration of *B. cereus* spores behaviour during 3 h incubation at 30ºC. Control growth in BHI (closed square), tempe 1 g l^{-1} fermented for 48 h (closed circle) and tempe 10 g l^{-1} fermented for 48 h (open square).

After germination, the spores in BHI with or without added cooked beans extracts showed a normal pattern of outgrowth of vegetative cells. Spores germinated in BHI with added tempe extract showed a long "lag" phase after germination, followed by an increase of optical density after 8 h indicating resumption of growth. The two tempe concentrations showed similar growth inhibition of *B. cereus*. Figure 3B presents development of viable counts of *B. cereus* after inoculation of spores in BHI with or without added tempe extract. A reduction of viable counts of 3.7 log CFU ml^{-1} within 2 h was observed, with the fastest decrease of viable cells within the first 30 min. This experiment also revealed similar effects of the different concentrations of tempe extracts used.

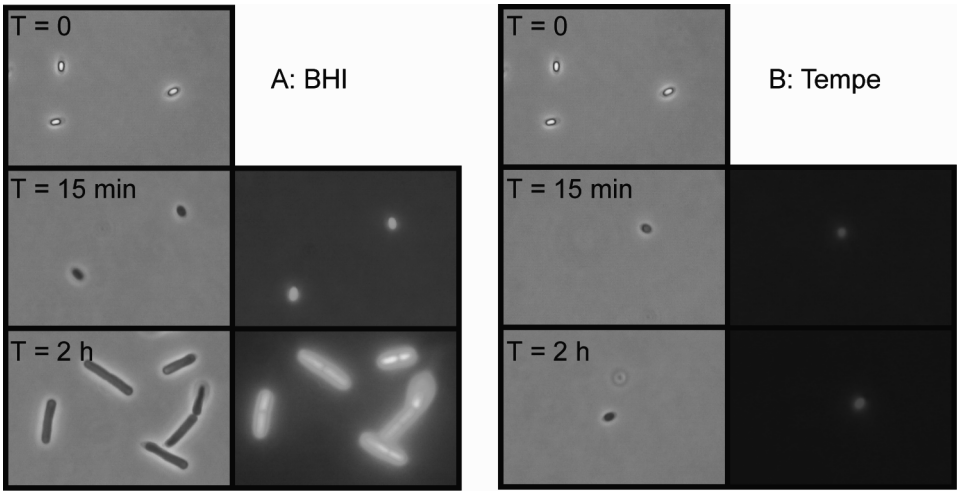

Figure 4: Spore germination and outgrowth in Brain Heart Infusion (BHI), as affected by added tempe extracts. Phase contrast (left) and fluorescence (right) microscopy photographs of *B. cereus* ATCC 14579 spores in BHI (A) and BHI supplemented with 1 g l^{-1} tempe extract (B) after 0 min, 15 min and 2 h. For fluorescence microscopy the green fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI) were used.

Spore germination and outgrowth in BHI with or without added tempe extract was monitored using phase contrast and fluorescence microscopy (figure 4). After 15 min of incubation with and without tempe extract, dormant spores changed from phase bright to the phase dark stage. As was also shown in the OD-measurement (figure 3A), tempe did not hamper the first stage in spore germination. Phase-contrast and fluorescence microscopy showed that the phase dark spores with tempe were stained

red, whereas the spores germinated in normal BHI were stained green. The red color indicates the red-fluorescent membrane-impermeant dye PI to have entered the germinated spores signifying membrane damage by the incubation with tempe extract. After 2 h of incubation in BHI with added tempe extract, all spores still remained phase dark and appeared red-fluorescent (PI-stained) after live-dead staining, whereas the spores that germinated in BHI had developed rod-shaped cells that appeared green-fluorescent (SYTO 9-stained) after staining.

Tempe antibacterial spectrum

Antibacterial activity of tempe was tested against different *Bacillus spp.* at two extraction pH values: pH 7 and pH 8. Table 1 shows tempe to exert an antibacterial effect on all tested *B. cereus* strains and also on *B. weihenstephanensis* and *B. subtilis*. Tempe extracts at pH 8 showed a longer growth delay than extracts obtained at pH 7; extracts of tempe fermented for 24 h showed a longer growth delay than the tempe fermented for 48 h for all tested strains. No growth of any tested *Bacillus* strain occurred within the 22 h of the measurement in extracts of tempe fermented for 24 h and adjusted to pH 8.

Table 1: Growth delay (h) based on optical density of *Bacillus* strains exposed to tempe extracts.

	Growth delay (h)			
	Tempe, 24 h[1]		Tempe, 48 h[1]	
Strain	pH 7[2]	pH 8[2]	pH 7[2]	pH 8[2]
B. cereus (ATCC 14579)	9.2 ± 0.4	> 16.6	6.3 ± 0.1	> 16.6
B. cereus (B437)	11.7 ± 4.4	> 15.1	5.3 ± 0.4	13.0 ± 2.9
B. cereus (PAL 20)	> 16.7	> 16.6	10.8 ± 1.1	13.9 ± 3.9
B. cereus (PAL 28)	> 16.8	> 16.7	6.6 ± 0.3	10.6 ± 0.0
B. cereus (55)	13.7 ± 0.4	> 16.4	7.1 ± 0.3	> 16.4
B. weihenstephanensis (DSM 11821T)	12.6 ± 3.1	> 15.6	7.5 ± 0.0	13.9 ± 2.5
B. cereus (ATCC 10987)	> 16.6	> 16.3	15.2 ± 2.0	> 16.3
B. subtilis	10.9 ± 2.2	> 12.5	> 12.5	> 12.5

Growth delay is expressed as mean ± standard deviation.

[1]Tempe extracts 10 g l^{-1} in BHI of 24-h and 48-h fermented tempe.

[2] Growth delay measured at pH 7 and pH 8.

>means that the growth delay is longer than the total measurement period (22 h).

Characterization of the inhibitory agent

Tempe extracts were tested for their antibacterial activity after pre-incubation at different pH values, exposure to a range of enzymes and after heat treatment (table 2). After pH adjustment of the tempe extracts, a growth delay of *B. cereus* could only be observed at pH 6 and higher, with highest inhibition activity at pH 8 and 9.

After treating tempe extracts with Pronase E or Proteinase K, all antibacterial activity was lost. The activity was partly susceptible to the Protease type XIII and resistant to Trypsin and α-Chymotrypsin.

After heat treatment exceeding 60°C, the antibacterial activity was gradually lost. Heating up to 60°C resulted in an activity loss of about 20% and 60% in extracts of tempe fermented for 24 h and 48 h, respectively. Cooking led to complete loss of activity for the extract of 48-h-fermented tempe, whereas the 24-h sample retained 30% of its activity. Notably, the 24-h-fermented tempe was more active than the 48-h-fermented tempe, and was also more resistant to low pH and higher temperatures.

Discussion

This study describes the antibacterial effect of soya bean extracts of intermediate stages of processing and fermentation of tempe on *B. cereus* vegetative cells and spores. The antibacterial activity was found to be specific for tempe extracts and was sensitive to heat, low pH and proteases, indicating its proteinaceous nature.

Two antibacterial activity assays were performed, using optical density monitoring and viable count enumeration (figure 1). Both showed a rapid bactericidal effect by a long growth delay of 12 h and a reduction in viable count of 3.7 log CFU ml^{-1} of the *B. cereus* cells after addition of tempe extract. After several hours a re-growth of the bacteria was observed in both experiments and could be explained by either degradation of the active component, or a surviving subpopulation. Conceivably, enzymes produced by *B. cereus* or produced by the mould during fermentation could be responsible for this reduction of antibacterial activity of the tempe extracts. Indeed, when *B. cereus* overnight culture supernatants were added to tempe extracts, the antibacterial activity of tempe was reduced to 10% of its initial value (data not shown). It is known that *Bacillus spp*. can produce a diversity of soluble extracellular enzymes (Priest, 1977), including proteases that may inactivate the active component(s).

Experiments with *B. cereus* spores showed that tempe could inactivate spores upon their germination. The optical density data (figure 3A) demonstrate the transition of phase bright spores into phase dark spores, followed by outgrowth of the vegetative

Table 2: Effect of pH, enzyme and heat treatments on growth inhibition activity of tempe extracts against *B. cereus* ATCC 14579.

Treatment	Activity (%)	
	Tempe, 24h[1]	Tempe, 48h[1]
pH		
2	0.4 ± 0.8	ND
3	2.0 ± 0.8	ND
4	0.5 ± 0.8	ND
5	17.3 ± 1.7	15.8 ± 4.5
6	54.2 ± 2.3	16.9 ± 1.3
7.1 (control)	100 ± 7.8	100 ± 4.7
8	> 100[2]	> 100[2]
9	> 100[2]	> 100[2]
Enzyme		
No enzyme (control)	nd	100 ± 14.1
Pronase E	nd	-1.8 ± 0.8
Proteinase K	nd	-3.5 ± 0.3
Protease XIII	nd	67.6 ± 5.4
Chymotrypsin	nd	101.0 ± 9.2
Trypsin	nd	> 100[2]
Heat (ºC, 20 min)		
4	100.0 ± 5.3	100 ± 12.1
20	97.6 ± 5.0	99.5 ± 12.6
30	106.7 ± 4.9	101.7 ± 10.4
40	118.2 ± 6.0	99.9 ± 12.8
50	131.9 ± 6.2	73.9 ± 10.4
60	83.9 ± 5.5	42.3 ± 9.1
70	47.9 ± 4.9	28.8 ± 8.3
80	41.1 ± 5.5	18.1 ± 8.5
90	33.6 ± 6.9	1.8 ± 7.2
100	32.2 ± 6.5	-4.0 ± 7.1

Activity is expressed in % activity after treatment compared with the tempe without any treatment in the specific experiment. Activity is expressed as mean ± standard deviation.
nd, not determined.
[1]Tempe extracts 10 g l^{-1} of 24h and 48h fermented tempe.
[2]No growth observed within measurement period.

cells. The spores in BHI with added tempe extract showed a growth delay of around 6 h compared with the spores growing in control BHI, which suggests that only a small

surviving subpopulation of spores was able to grow. Fluorescence microscopy experiments showed that after addition of tempe extract (figure 4B) the phase dark spores (after germination) were stained red by PI, which indicates that tempe extracts induced considerable membrane damage to the germinated spores as was observed also with vegetative cells. The effect of tempe on dormant spores could not be measured as the tempe extracts already triggered the germination of the spores to the phase dark stage. Mechanistic information about effects of antibacterial compounds against spores is limited. A recent study, concerning the well known antibacterial compound nisin, showed nisin to act against germinated spores of *Bacillus anthracis* and not against dormant spores. The mode of action of nisin responsible for outgrowth inhibition of *B. anthracis* spores appeared to involve inhibition of the oxidative metabolism and dissipation of the membrane potential, indicative of disruption of membrane integrity (Gut *et al.*, 2008). Although our results also point to membrane targeting of the antibacterial activity of tempe extracts, the active component is different from nisin, based on its sensitivity to heat and low pH, and its inactivation by a range of proteases.

In the research of Wang *et al.* (1969) and Kobayasi *et al.* (1992) antibacterial activity of *Rhizopus oligosporus* grown in culture broth was investigated. In contrast, we were not able to determine antibacterial activity of *R. microsporus* grown in culture broth, even when it had been grown on cooked soya bean agar. However, after the fermentation of *R. microsporus* on soya beans a high bactericidal activity was observed. It is conceivable that the antibacterial activity is produced by microorganisms present in tempe, i.e. *R. microsporus* and LAB bacteria. This indicates on the one hand, that the antibacterial activity is only produced *in situ*, but on the other hand, it may indicate that degradation products of soya proteins are responsible for the antibacterial effect of tempe, thus pointing to an indirect role of the indicated microorganisms. Notably, for milk-derived peptides such as bovine lactoferricin from lactoferrin and several peptides from casein, a strong antimicrobial activity was observed after microbial hydrolysis (López-Expósito and Recio, 2008). Further characterization of the antimicrobial activity will shed light on this hypothesis.

To conclude, tempe extracts display antibacterial activity against *B. cereus* cells and spores by targeting the membrane of the bacteria. This bactericidal activity is released during fermentation of soya beans only, whereas extracts of raw, soaked and cooked soya beans, and *R. microsporus* grown in culture media did not give any antibacterial activity. The tempe antibacterial activity was found to be heat, low pH and protease sensitive, suggesting its proteinaceous nature. Further research will be required to

identify the chemical nature of the antibacterial component(s) and its bio-functionality in terms of food preservation and pathogen control.

Acknowledgements

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Chapter 4

Bioactivity of tempe by inhibiting adhesion of ETEC to intestinal cells, as influenced by fermentation substrates and starter pure cultures

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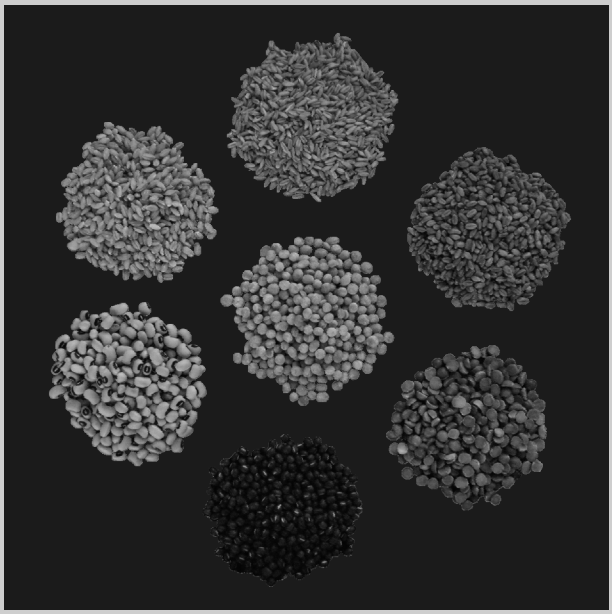

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Abstract

Soya bean tempe is known for its bioactivity in reducing the severity of diarrhoea in piglets. This bioactivity is caused by an inhibition of the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to intestinal cells. In this paper, we assessed the bioactive effect of soya tempe on a range of ETEC target strains, as well as the effect of a range of cereal and leguminous substrates and starter pure cultures.

Soya bean tempe extracts strongly inhibited the adhesion of ETEC strains tested. All tempe made from other leguminous seeds were as bioactive as soya bean tempe, whereas tempe made from cereals showed no bioactivity. Using soya beans as substrate, fermentation with several fungi (*Mucor*, *Rhizopus* spp. and yeasts) as well as *Bacillus* spp. resulted in bioactive tempe, whereas fermentation with lactobacilli showed no bioactivity.

The active component is released or formed during the fermentation and is not present in microbial biomass and only partly in unfermented substrates. The bioactivity being not specific for a single ETEC strain, makes the bioactive tempe relevant for applications in animal husbandry.

Introduction

Tempe is a fungal fermented food originating from Indonesia, which is made mostly from soya beans through fermentation with *Rhizopus* spp. (Nout and Kiers, 2005). In the final product the cottony mycelium binds the soya beans together to a compact cake. During fermentation of soya beans, a range of fungal enzymes is produced including proteases, lipases, carbohydrases and phytases. These enzymes degrade macromolecules into lower molecular weight substances, thus partly solubilizing the cell walls and intracellular material, leading to an increased nutritional quality and digestibility (Nout and Kiers, 2005). Previous research showed that tempe made from soya beans fermented with *Rhizopus microsporus*, can reduce the severity of diarrhoea in piglets (Kiers *et al.*, 2003). This effect of soya bean tempe is obtained by inhibition of the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to intestinal brush border cells (Roubos-van den Hil *et al.*, 2009). Reduced adhesion of ETEC strains to intestinal cells results in reduced colonization and enterotoxin production, manifested by a lower diarrhoeal incidence (Nataro and Kaper, 1998).

The present work was performed to determine whether the use of different substrates and starter pure cultures do influence this bioactivity of tempe. In addition it is determined whether this bioactivity is generic for other ETEC strains. This would give more information about the bioactive component and applicability of the tempe bioactivity.

Previous research (Kiers *et al.*, 2002; Roubos-van den Hil *et al.*, 2009) was performed using a single ETEC target strain as a model to investigate adhesion inhibition. No published data on the inhibition of adhesion of different ETEC strains is available. Therefore, the first aim of the present study was to test tempe bioactivity against a wide range of enterotoxigenic *Escherichia coli* (ETEC) bacteria isolated from piglets with diarrhoea.

Tempe is considered as the collective name for various pulses and cereals fermented with a fungi belonging to the *Rhizopus* genus. Traditionally, tempe is made from soya beans and, therefore, most research is done with soya bean tempe. However, other substrates such as barley (Eklund-Jonsson *et al.*, 2006; Feng *et al.*, 2007a), chick pea (Ashenafi and Busse, 1991), cowpea (Kiers *et al.*, 2000a; Egounlety, 2001), groundbean (Egounlety, 2001), horse bean (Ashenafi and Busse, 1991), pea (Ashenafi and Busse, 1991), oats (Eklund-Jonsson *et al.*, 2006), sorghum (Mugula and Lyimo, 2000) and wheat (Hachmeister and Fung, 1993) were also reported to be suitable substrates to produce tempe. The impact of using different substrates on the adhesion

inhibition was not investigated before. Hence, the second aim of this research was to determine the bioactivity of tempe prepared with different leguminous and cereal substrates. Thereby several fermentation parameters were determined to confirm a successful fermentation of the different products into a tempe product.
A diverse range of microorganisms may be encountered in tempe, including filamentous fungi involved in the inoculation and fermentation of tempe, as well as high levels of bacteria and yeasts (Nout and Rombouts, 1990). Research on the microbial quality of commercial tempe in The Netherlands showed that most samples had an aerobic plate count exceeding 10^7 CFU g^{-1}, with lactic acid bacteria over 10^7 CFU g^{-1} and yeast levels higher than 10^5 CFU g^{-1} in 69% of the samples (Samson *et al.*, 1987). While the contribution of these bacteria and yeasts to the properties of tempe is only partly understood, they do play a role in flavour development and chemical substrate modification (Nout and Rombouts, 1990). Lactic acid bacteria were shown to play a role in acidification of the soya beans during soaking, thereby preventing the growth of spoilage causing microorganisms (Nout *et al.*, 1987). In order to research the origin and formation of the bioactive principle, the third aim of this research was to test the bioactivity of soya beans inoculated with different microorganisms (isolated from tempe and similar fermented products) and to monitor their growth during fermentation.

Materials and methods

Microorganisms

Ten ETEC K88 strains and one ETEC K91 strain (for serotypes see table 1) were grown in brain heart infusion (BHI) broth (Becton Dickinson, 237500) at 37ºC overnight. The strains were provided by the collection of the Animal Science Group, Lelystad, Wageningen University an Research Centre, The Netherlands. The cultures were centrifuged (3000 *g*, 10 min) and washed twice with phosphate-buffered saline (PBS) (NaCl 136.89 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 2.79 mM, pH 7.2), followed by suspending the pellets in PBS, to an optical density of 0.75 corresponding with approximately 10^9 CFU ml^{-1}.
For fermentation, 2 *Lactobacillus*, 8 mould, 3 *Bacillus* and 7 yeast strains from the Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands were used (table 2). *Lactobacillus* strains were maintained on de Man, Rogosa and Sharpe (MRS) agar plates (Becton Dickinson, 288130). Two days before use in experiments the strains were inoculated in MRS broth and incubated at 30ºC.

Table 1: ETEC strains.

Strain no[1]	Serotype	Toxins
ID 1000	O149:K91:K88^{ac}	LT, STb
ID 1002	O149:K91:K88^{ac}	LT, STb
ID 1006	O149:K91:K88^{ac}	LT, STb
ID 1008	O149:K91:K88^{ac}	LT, STb
ID 1009	O8:K87:K88^{ac}	LT, STb
ID 1010	O138:K81:K88^{ac}	LT, STb
ID 1012	O8:K87:K88^{ac}	LT, STb
ID 1018	O138:K81:K88^{ac}	LT, STb
ID 1022	O138:K81:K88 ^{ac}	LT, STb
ID 1063	O8:K87:K88^{ac}	LT, STb
ID 1084	O149:K91	

[1] Strains were obtained from the collection of the Animal Sciences Group, Wageningen University and Research Centre, Lelystad, The Netherlands.

The cultures were then washed and diluted in peptone physiological salt solution (PPS), containing neutralized bacteriological peptone 1 g l^{-1} (Oxoid, LP34) and NaCl 8.5 g l^{-1}, to approximately 10^6 CFU ml^{-1}. *Bacillus* strains were maintained on BHI agar. One day before the start of the experiment the strains were inoculated in BHI broth and incubated at 30ºC while shaking at 200 rpm. The cultures were washed and diluted in PPS to approximately 10^6 CFU ml^{-1}. Yeast strains were maintained on Malt Extract Agar (MEA) (Oxoid, CM59). Two days before the start of the experiment strains were inoculated in Malt Extract Broth (MEB) (Oxoid, CM57) and incubated at 30ºC while shaking at 200 rpm. The cultures were washed and diluted in PPS to approximately 10^6 CFU ml^{-1}. Moulds were maintained on MEA slopes; 7 days before the start of the experiment they were inoculated on fresh MEA slopes and incubated at 30ºC. A spore suspension was prepared by adding 10 ml PPS per slope and releasing the sporangia to obtain a suspension with a concentration of 10^5-10^6 CFU ml^{-1}.

Tempe fermentation with different substrates and *Rhizopus* spp.

Soya beans (yellow-skinned variety used for tempe making (USA), normal and organically farmed), cowpea (Benin, West Africa), green pea, red bean, wheat, oat and barley (all from local stores the Netherlands) were used as substrates for fermentation. Substrates were soaked overnight in tap water at 30ºC. In order to achieve an accelerated lactic acid fermentation during this soaking step, the soak water had been inoculated with naturally acidified soaking water ("backslop") (Nout *et al.*, 1987).

Table 2: Microorganisms used for soya bean fermentation.

Strain no.[1]	Name	Isolated from
Lactobacillus		
LU 848	*Lactobacillus plantarum*	Soya soak water
LU 852	*Lactobacillus plantarum*	Soya soak water
Bacillus		
LU 810	*Bacillus licheniformis*	
LU 812	*Bacillus pumilus*	
LU 814	*Bacillus subtilis*	
Yeasts		
LU 121	*Candida intermedia*	Tempe
LU 502	*Pichia guilliermondii*	Tempe
LU 677	*Saccharomycopsis fibuligera*	Ragi
LU 692	*Trichosporon beigelii*	Tempe
LU 1251	*Saccharomyces cerevisiae*	Rice wine
LU 1253	*Candida glabrata*	Rice wine
Moulds		
LU 361	*Mucor circinelloides (f. circ.)*	Tempe
LU 365	*Mucor indicus*	Tempe
LU 573	*Rhizopus microsporus*	Tempe
LU 575	*Rhizopus oligosporus*	Tempe
LU 581	*Rhizopus oryzae*	Tempe
LU 2036	*Rhizopus microsporus*	Sufu
LU 2040	*Rhizopus oligosporus*	Rice wine starter
LU 2041	*Rhizopus oryzae*	Rice wine starter

[1] Strains were obtained from the Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands.

Next, the substrates were rinsed with tap water and cooked in fresh tap water for 20 min at a substrate:water ratio of 1:3 (w/v). Subsequently, the substrates were cooled and surface dried at room temperature, and were spread out on mesh trays for about 1 h. For the fungal inoculation of the substrates a sporangiospore suspension from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU 573) was used. After inoculation with the spore suspension (10 ml kg^{-1}), the substrates (batches of 450 g) were packed into hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated for 48 h at 30ºC. Cooked and fermented substrates were stored and used for analyses.

Soya bean fermentation with different microbial inoculants

Soya beans were soaked overnight in tap water at 4ºC (bean:water ratio 1:3 (w/v)) to avoid fermentative acidification. Beans were washed and subsequently cooked for 20 min in fresh tap water. After cooking, the beans were cooled and surface dried at room temperature. Next, 100 g of beans were transferred into glass jars and sterilized at 121ºC for 30 min. After cooling to room temperature the beans were inoculated with 5 ml of the diluted microbial cultures (table 2). After mixing, the beans were incubated aerobically for 48 h at 30ºC.

pH measurement and microbiological analyses

Cooked and fermented samples (5 g) were mixed with 45 ml PPS and homogenized in a stomacher (Seward stomacher circulator 400; 1 min; 200 rpm). The pH was measured in this suspension with a pH meter (WTW digital pH meter 525 with electrode Sentix 4.1). Further decimal dilutions in PPS were prepared and plated on appropriate growth agars. Fermented samples from different substrates were plated on plate count agar (PCA) (Oxoid, CM325) for total viable count and on MRS-agar for lactic acid bacteria (LAB) count. The beans incubated with different microorganisms were plated on the same media as had been used for their cultivation. Plates were incubated for 24 h at 30ºC; MRS-plates were incubated anaerobically. The development of the mould mycelium and appearance of the tempe cakes after incubation were assessed visually.

Dry matter content and solubility

Dry matter content was determined by freeze drying 50 g of the samples. Freeze-dried samples were ground to a fine flour (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) passing through a 0.5 mm sieve. Solubility was arbitrarily defined as the dissolving capacity of 5 g sample in 100 ml water using subsequent extractions. It was quantified by suspending 5 g of freeze-dried sample in 50 ml distilled water and incubating at 37ºC for 30 min with continuous shaking. After centrifugation (2600 *g*; 15 min, 4ºC), supernatants were collected and pellets were re-suspended with 20 ml of distilled water and centrifuged. This re-suspension step was repeated once. The supernatants collected from the three centrifugation steps were pooled and adjusted to 100 ml. Ten ml of this solution was oven-dried at 80ºC for 24 h, from which dissolved dry matter was calculated. The remaining 90 ml was freeze-dried and used for subsequent experiments.

Protein degradation

Total nitrogen contents of the various samples were determined in duplicate by the Dumas method using an NA2100 Nitrogen and Protein Analyzer (CE INSTRUMENTS) according to the manufacturer's instructions; methionine was used as a standard. The degradation of proteins was measured by formol titration of terminal (free) amino nitrogen. Freeze-dried ground samples were weighed (1.0 g) and suspended in 25 ml distilled water with continuous stirring. The pH was adjusted to 8.5 with 0.1 M NaOH and 5 ml formaldehyde (35%) solution (pH=8.5) was added and left for 2 min for the reaction to take place. The solution was titrated back to pH 8.5 with 0.1 M NaOH. The used volume in the titration was directly proportional to the amount of free amino nitrogen (Han *et al.*, 1999). The formol value expressed as free amino nitrogen gives an indication of the hydrolysis of proteins.

Reducing sugars

Reducing sugars were determined according to Nelson-Somogyi (Green *et al.*, 1989). Briefly, 1.0 g of freeze-dried ground samples was suspended in 25 ml distilled water with continuous stirring for 1 h. Samples (450 µl) were mixed with 450 µl copper reagent, consisting of 4 parts of K-Na-tartrate:Na_2CO_3:Na_2SO_4:$NaHCO_3$ (1:2:12:1.3) and 1 part of $CuSO_4 \cdot 5H_2O$:Na_2SO_4 (1:9). The first reagent was prepared by boiling to completely dissolve the components. The samples were cooked for 10 min and cooled to room temperature. Arsenomolybdate reagent was prepared by mixing 26.5 g ammoniummolybdate in 450 ml distilled water with addition of 21 ml concentrated H_2SO_4 and 3 g of $Na_2HAsO_4 \cdot 7H_2O$ in 25 ml distilled water and this solution was incubated for 24 h at 37°C. Prior to use, 1 part of this solution was mixed with 2 parts of 1.5 M H_2SO_4 and 450 µl of this reagent was added to the samples and mixed. After 30 min 3 ml of distilled water was added and the absorption was measured at 520 nm. For the calibration glucose was used in a concentration of 0-150 µg ml^{-1}.

Bioactivity assay

Bioactivity of the different products was measured with the brush border adhesion assay. Brush border cells were isolated from the jejunum of a K88-receptor positive, early weaned six week old piglets were used according to Sellwood *et al*., (1975). The brush border cells were exposed to ETEC K88 (ID1000) to confirm positive receptor status; brush borders that bound more than 8 ETEC K88 per brush border cell were recorded as K88-positive. In our experiments, we also used a non-adhering *E. coli* (O149:K91) strain ID1084 as a negative non-adhering control.

Freeze-dried soluble extracts (10 mg) were dissolved in 1 ml PBS, mixed in a head-over-tail rotator for 1 h and centrifuged (10,000 *g*, 10 min, 20ºC). Supernatants were diluted to respectively 2.5 g l^{-1} and 1 g l^{-1}, and 30 µl was mixed with 30 µl of ETEC K88 suspension and 30 µl of brush border cell suspensions. The mixture was incubated at room temperature with continuous gentle shaking (100 min^{-1}) in a plate shaker (Plate shaker KL2, Edmund Bühler GmbH, Hechingen, Germany) for 1 h. The number of bacterial cells, adhering to 12 individual brush border cells, was determined by phase contrast microscopy (magnification 1000x). The proportion of adhesion was calculated as the average number of ETEC K88 per brush border cell, relatively to the adhesion with the positive control.

Statistical analyses

The significance of the bioactivity experiments was evaluated by comparing means using one or two-way ANOVA, followed by the Bonferroni post-test. Results were expressed as mean ± SEM and differences were considered significant at $P < 0.05$. Statistics were performed with Graphpad Prism version 4.03 for Windows (Graphpad Software, San Diego, CA, USA).

Results

Bioactivity of soya bean tempe towards different ETEC bacteria

Several strains of ETEC bacteria, isolated from piglets suffering from diarrhoea found in different farms in The Netherlands were used (table 1). These strains were tested for their adherence to brush border cells as represented in figure 1. Strain ID 1000 and ID 1084 were used as positive and negative control strains, respectively (Roubos-van den Hil *et al.*, 2009). Incubation of brush border cells with strain ID 1000 resulted in an adhesion of 10.3 ± 0.66 (mean ± SEM) ETEC cells to one brush border cell. This value was used as a reference representing 100% adherence. Strain ID 1084 is known as a negative strain, which means that the bacteria were not capable of adhering to brush border cells. All other tested ETEC strains adhered to the brush border cells, except strain 1012. Among the other strains differences were observed in the number of ETEC adhering to a brush border cell, but incubation of the brush border cells in presence of tempe and these ETEC bacteria always resulted in very low adhesion values (figure 1). The resulting adhesion values were of the same order as observed previously for strain ID 1000 (Roubos-van den Hil *et al.*, 2009).

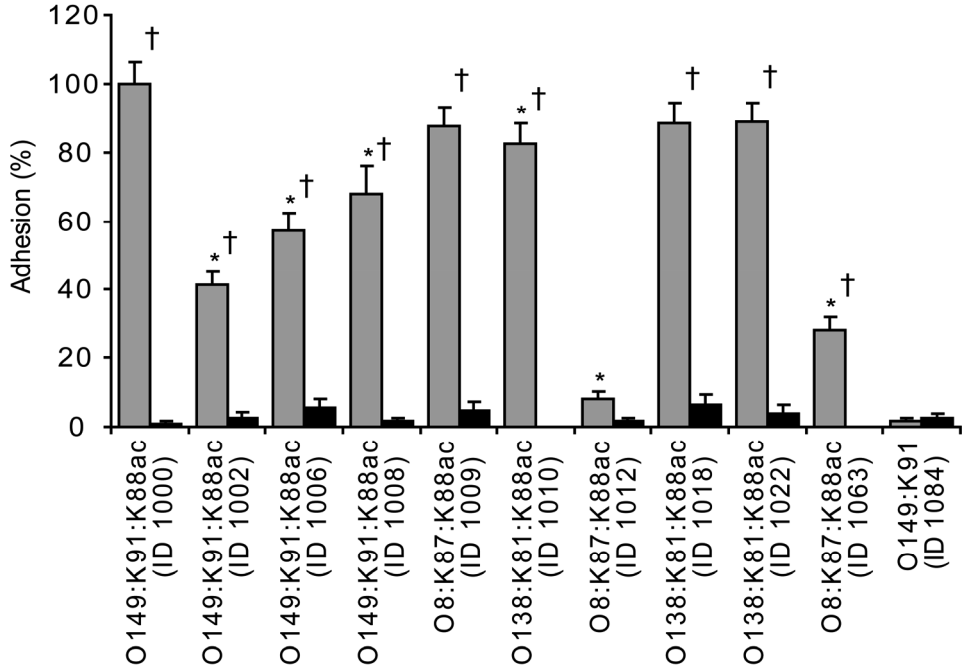

Figure 1: Adhesion of different ETEC strains to piglet brush border cells. Gray bars represent adhesion without addition and black bars with addition of tempe extract (2.5 g l^{-1}). Bars represent mean values, expressed as % adhesion compared to the positive control (strain ID 1000) of 12 individual brush borders. Error bars represent SEM. Bars with asterisk (*) differ significantly from strain ID 1000. Bars with † represent a significant effect of addition of tempe extract.

Monitoring tempe fermentation from different substrates

After fermentation of different substrates with *R. microsporus* (LU 573), all substrates except red beans and wheat were fully fermented, i.e. overgrown and penetrated by fungal mycelium. The visual appearance of the fermented substrates was a dense cotton mycelium that bound the individual legumes or cereals to a cake-like product. Also, the smell of these products was fresh and typical of good quality tempe. In the red bean and wheat product the mould had not fully penetrated the centre of the product, but mainly grown at the outside, which resulted in a loose cake, which was easy to break.

Table 3 shows the fermentation parameters that were analyzed. During cooking the pH had increased slightly (data not shown) and after 48 h of fermentation the pH was increased in all of the fermented substrates except in oat. Solubility increased in all fermented substrates, with a maximum of 7 times the initial amount of soluble material

Table 3: Fermentation characteristics of leguminous and cereal grains fermented with *Rhizopus microsporus* (LU 573).

		pH	solubility (g/ 100 g dry matter)	Amino nitrogen (mmol free amino group / 100 g dry matter)	protein (g / 100 g dry matter)	Reducing sugars (mg / 100 g dry matter)	LAB[1] log CFU/ g	TVC[2] log CFU/ g
Soya	Cooked	4.6	7.0	15.0	41.8	192.1	2.2	2.3
	Fermented (48 h)	6.0	19.0	49.4	43.8	464.8	8.7	8.8
Organic soya	Cooked	4.7	4.0	17.1	48.3	218.5	2.3	2.3
	Fermented (48 h)	5.8	15.0	44.7	48.2	517.6	9.2	9.2
Cowpea	Cooked	5.0	3.0	10.7	22.2	180.6	3.5	3.3
	Fermented (48 h)	5.8	12.0	23.7	25.9	818.5	8.5	8.5
Green pea	Cooked	5.2	2.0	11.3	22.5	275.9	<2	<2
	Fermented (48 h)	5.9	14.0	23.2	24.6	857.4	6.6	6.8
Red bean	Cooked	6.7	8.0	10.0	22.6	50.0	<2	3.6
	Fermented (48 h)	7.0	14.0	15.6	22.9	675.0	6.4	6.5
Wheat	Cooked	5.5	3.0	3.3	13.6	325.9	<2	<2
	Fermented (48 h)	6.5	12.0	5.8	13.3	855.1	6.2	6.7
Oat	Cooked	5.2	5.0	5.3	15.0	194.9	3.3	3.7
	Fermented (48 h)	5.1	15.0	11.4	18.8	970.8	6.2	6.5
Barley	Cooked	4.5	3.0	2.1	9.7	25.9	2.1	2.2
	Fermented (48 h)	5.3	9.0	8.1	11.2	887.5	6.3	7.6

[1] LAB: Lactic Acid Bacteria

[2] TVC: Total Viable Count of mesophilic aerobic bacteria.

in fermented green peas. The amount of free amino nitrogen was increased for all substrates during fermentation. In the legumes the amount of free amino nitrogen before and after fermentation was higher than in the cereals, but also the amount of proteins in the legume substrates was higher. All substrates showed an increase of at least 2 times the amount of free amino nitrogen, except the wheat and red bean tempe, which was in accordance with our expectations based on the poor mycelium development in these products. The level of reducing sugars also increased strongly during tempe fermentation, especially in the non-soya substrates.
Microbiological observations during the fermentation showed strong growth of LAB, up to log 9 CFU g^{-1}, in the two soya products and the cowpea product. In the other substrates also growth of LAB and total mesophilic aerobic bacteria was observed, but values were lower, *i.e*. log 6-7 CFU g^{-1}.

Bioactivity of tempe prepared with different substrates
Bioactivity of the different cooked substrates are shown in figure 2. Whereas some significant extent of adhesion inhibition was observed for the cooked legumes, the cooked cereal extracts did not inhibit adherence significantly. After fermentation the bioactivity of the tempe extracts increased significantly with all legume substrates, whereas the fermented cereals still showed no activity. Soya, cowpea and green pea extracts inhibited the adhesion to values lower than 20% of the positive control. The red beans substrate showed some adhesion inhibition, but this activity was not increased as a result of fermentation, which could very well be related to the incomplete fermentation of this substrate.

Soya bean fermentation with different microorganisms
Cooked and sterilized soya beans were inoculated with pure starter cultures of different microorganisms, namely 2 *Lactobacillus* spp., 3 *Bacillus* spp., 6 yeasts and 8 mould strains (table 2). After a 48 h incubation period, the fermented samples were analyzed and compared with the cooked substrates, as shown in table 4.
The two tested LAB strains, that had been isolated from soya soaking water, grew to 9 log CFU g^{-1}, a level that was also observed in regular tempe (table 3). During incubation the pH decreased and a distinct sour odour was observed. All reducing sugars were utilized, but the levels of free amino nitrogen did not change.
After incubation with the *Bacillus* spp., soya beans were sticky and an ammoniacal odour was observed. The *Bacillus* spp. were able to grow up to 10 log CFU g^{-1}, while the pH and levels of free amino nitrogen and reducing sugars had increased.

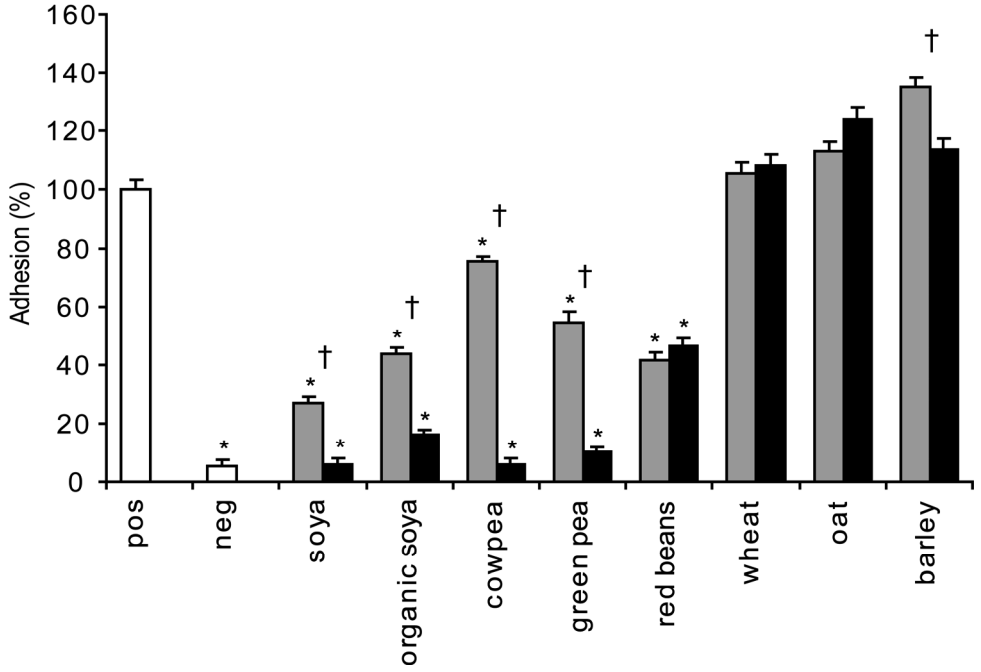

Figure 2: Adhesion of ETEC K88 to brush border cells with addition of extracts of cooked and fermented substrates. White bars represent controls without any addition of extract; Grey bars represent extracts of cooked legumes and cereals (2.5 g l^{-1}); Black bars represent the fermented legumes and cereals (2.5 g l^{-1}). Bars represents mean values, expressed as % adhesion compared to the positive control of 12 individual brush borders. Error bars represent SEM. Bars with asterisk (*) inhibit adhesion significantly compared with the positive control. Bars with † represent a significant difference between cooked and fermented substrates.

All yeasts were able to grow after inoculation to 8-9 log CFU g^{-1}. In contrast to the other strains, the pH of soya beans incubated with *Saccharomyces cerevisiae* (LU 1251) and *Candida glabrata* (LU 1253) was not increased during fermentation. *Saccharomycopsis fibuligera* (LU 677) increased the levels of free amino nitrogen and reducing sugars. *Candida intermedia* (LU 121), *Trichosporon beigelii* (LU 692) and *S. cerevisiae* (LU 1251) caused an increase of reducing sugars. The other yeast strains (*Pichia guilliermondii* LU 502 and *C. glabrata* LU 1253) decreased the level of reducing sugars, but had no effect on free amino nitrogen levels. All mould strains were able to grow, and bound the soya beans together to a firm cake. During fermentation all moulds caused an increase of reducing sugars and free amino nitrogen levels.

Table 4: Fermentation characteristics of soya beans fermented with a range of bacteria, yeasts and moulds.

Sample type	pH	Amino nitrogen (mmol free amino group / 100 g dry matter)	Reducing sugars (mg / 100 g dry matter)	Inoculation (log CFU / g)	Incubated 48 h (log CFU / g)
Cooked	6.6	19.9	59.2	-	-
Lactobacillus					
LU 848[1]	5.1	18.4	0.0	5.3	9.3
LU 852	5.1	18.4	0.0	5.1	9.2
Bacillus					
LU 810	7.0	41.9	456.1	4.6	10.0
LU 812	6.7	26.7	411.7	4.5	9.8
LU 814	7.6	74.9	172.5	4.4	9.4
Yeasts					
LU 121	6.9	17.3	282.5	5.4	8.9
LU 502	6.9	12.9	0.0	6.3	9.5
LU 677	7.2	38.4	114.5	4.4	8.3
LU 692	6.8	19.3	150.6	3.7	7.7
LU 1251	6.3	15.8	232.6	5.6	8.7
LU 1253	6.5	20.6	7.4	5.5	8.5
Moulds					
LU 361	7.0	33.1	445.6	nd[2]	nd
LU 365	7.0	37.0	544.2	nd	nd
LU 573	6.7	51.3	205.0	nd	nd
LU 575	6.4	68.6	718.3	nd	nd
LU 581	6.5	52.6	537.8	nd	nd
LU 2036	7.0	54.7	125.1	nd	nd
LU 2040	7.0	63.1	235.5	nd	nd
LU 2041	6.6	60.1	515.7	nd	nd

[1] See Table 2 for names of microorganisms

[2] nd, not determined

Bioactivity of soya beans fermented with different microorganisms

The bioactivity of the fermented soya beans is shown in figure 3. All moulds and *Bacillus* fermented soya beans showed adhesion inhibition of more than 90%. *Lactobacillus* fermented soya beans caused no inhibition of adhesion. Of the yeast fermented soya beans only those with *S. fibuligera* (LU 677) and *T. beigelii* (LU 692) showed inhibition of adhesion.

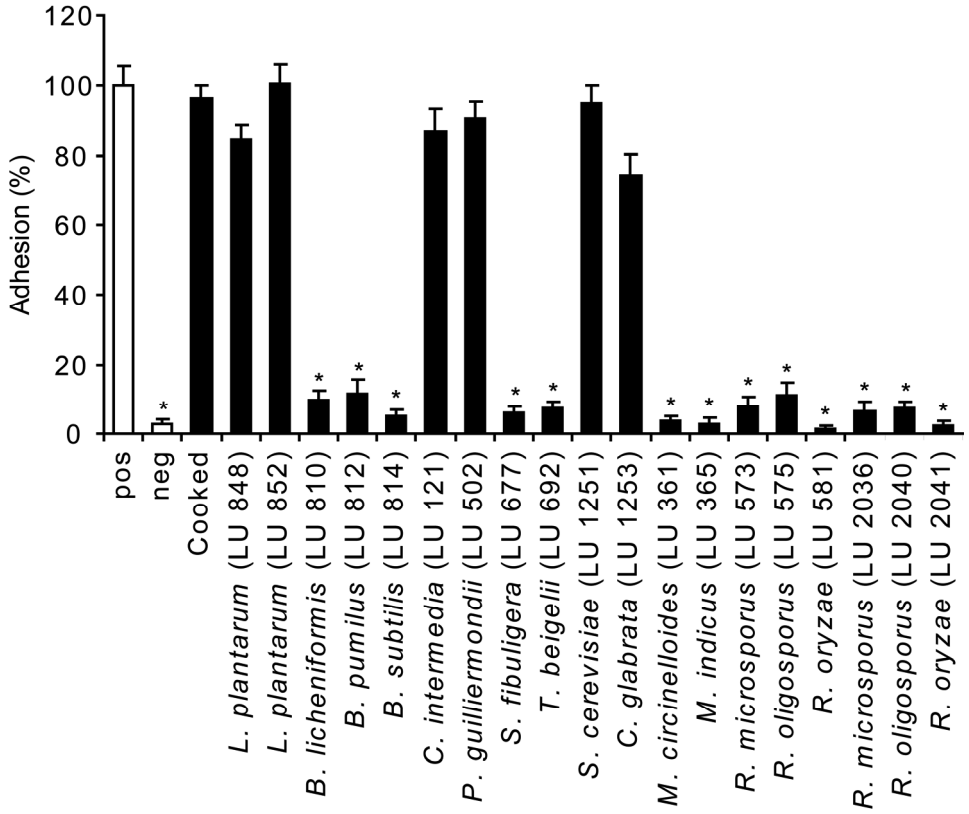

Figure 3: Activity of soya beans after incubation for 48 h with different microorganisms. White bars represent controls without any addition of extract; Black bars represent extracts of soya beans incubated with different microorganism (1 g l^{-1}). Bars represent mean values, expressed as % adhesion compared to the positive control of 12 measurements. Error bars represent SEM. Bars with asterisk (*) inhibit adhesion significantly compared with the positive control.

Discussion

Diarrhoeal disease in piglets is frequently due to infection by ETEC. It causes severe, watery diarrhoea especially in suckling and weaned piglets (Nagy and Fekete, 2005). ETEC is also recognized as one of the most frequent causes of childhood diarrhoea in developing countries, and of traveler's diarrhoea (Bhan, 2000). We tested the bioactivity of soya bean tempe on a range of ETEC strains of different serogroups with different fimbrial adhesins and observed that tempe extracts decrease the adhesion of most tested ETEC on brush border cells. Thus, tempe extracts can prevent intestinal cells being colonized by different strains of ETEC causing diarrhea in piglets.

During fermentation of soya beans with *Rhizopus* spp. diverse chemical modifications take place. During the soaking stage the pH of the soaked substrates was lowered by LAB. A high number of actively acidifying LAB mixed culture was obtained by using the back-slop technique (data not shown). The soaking step is important for the quality of the tempe, because it prevents the growth of spoilage causing bacteria (Nout *et al.*, 1987; Ashenafi and Busse, 1991). During cooking of beans the pH will start to increase, which continues during fermentation. This is a result of proteolysis and the release of ammonia following utilization of amino acids as carbon and energy source by the mould (Sarkar *et al.*, 1993). Oats did not show a pH increase, which can be due to an initial pH decrease during the first hours of fermentation during which sugars, and not proteins were used as substrates for growth. The solubility of all substrates increased during fermentation, which is due to the enzymatic degradation of macromolecules into substances of lower molecular weight with a higher solubility (Nout and Rombouts, 1990; De Reu *et al.*, 1995; Kiers *et al.*, 2000a). Enzymatic degradation was also evidenced by the increased levels of free amino groups and reducing sugars. Astuti (2000) showed that the effect of fermentation on total nitrogen content is neglible, but increases of free amino acids take place during fermentation. Higher levels of carbohydrates are found in the non-soya substrates, which upon degradation, result in higher values of reducing sugars. Research conducted elsewhere (Samson *et al.*, 1987; Mulyowidarso *et al.*, 1990; Ashenafi, 1994) indicated that in tempe total bacterial counts can reach 10^9 CFU g^{-1} and LAB can reach levels of 10^8 - 10^9 CFU g^{-1}, which is comparable with our observations in soya and cowpea tempe. The other substrates also supported microbial growth, but remarkably less, for example in barley tempe the counts were comparable with data reported by Feng *et al.,* (2005). Different levels of growth achieved in diverse substrates are assumedly associated with their individual nutrient composition.

Bioactivity was measured in all extracts of leguminous tempe and this activity increased during fermentation. In contrast, the cereal-derived tempe products showed no bioactivity at all. It appears that during fermentation an active component was released or formed by enzymatic breakdown from leguminous substrates. Mould biomass itself has no bioactivity since well-grown cereal-derived tempe lacked inhibition activity. The active component(s) is, therefore, specific for legumes. This could be related to the higher protein content or the protein composition in legume seeds. Another remarkable difference is the relatively high amount of isoflavones present in legumes, which are not (or at very low levels) present in cereals (Liggins *et al.*, 2002). Also the cell wall is different, monocotyledons (cereals) contain cellulose fibrils in close association with arabinoxylans, whereas dicotyledons (legumes) contain cellulose with pectin and xyloglucans (Harris and Smith, 2006).

Tempe is traditionally fermented with moulds, mainly *Rhizopus oryzae*, *Rhizopus oligosporus*, *R. microsporus* or *Mucor indicus,* of which the functionality has been reported earlier (Samson *et al.*, 1987; Nout and Kiers, 2005). In addition, tempe contains a range of bacteria such as LAB, *Bacillus* spp. and yeasts (Samson *et al.*, 1987; Nout and Rombouts, 1990), of which less is known about their function in the fermentation.

Before inoculation with the pure microbial strains the soya beans were soaked overnight at 4ºC instead of 30ºC to avoid fermentative acidification, since this acidification could influence the growth of acid sensitive strains. When testing the effect of tempe-derived pure microbial strains on soya beans, we observed that *Lactobacillus* spp. assimilated all available sugars to form lactic acid as indicated by concomitant pH decrease. The modification of soya beans with *Bacillus* spp. can be compared with that during the fermentation of *Kinema*, a traditional *Bacillus* fermented soya bean food in India and Nepal. These fermentations are characterized by extensive hydrolysis of proteins into amino acids, peptides and ammonia and a typical sticky appearance of the soya beans (Sarkar *et al.*, 1993; Nout *et al.*, 1998; Kiers *et al.*, 2000b). Our observations of *Bacillus* fermented soya beans were similar to *Kinema*.

The occurrence of yeasts has been reported in tempe products as yeasts can grow well in mixed microflora with lactic acid bacteria and filamentous fungi, but no yeast species are specifically associated with tempe (Samson *et al.*, 1987; Ashenafi and Busse, 1991; Feng *et al.*, 2007b). Our observations show that yeasts found in tempe were able to grow on soya beans and some yeasts were also able to interact (by degrading macromolecules) with the soya beans. The soya beans fermented with the

different mould strains were similar to tempe prepared following the usual process, confirming that tempe can be made without co-inoculants.
All *Bacillus* spp., yeast strains LU 677 and LU 692 and all tested mould strains caused inhibition of the ETEC adhesion after incubation with soya beans. Thus, activity was not related to a specific microorganism, but instead the degradation of certain macromolecules is needed to release or form bioactive component(s) from the soya beans.
In conclusion, tempe derived from leguminous seeds is bioactive, i.e. reduces adhesion of ETEC to piglet brush border cells, whereas tempe derived from cereals is inactive. The bioactive component(s) is released or formed during fermentation from leguminous matter. The capability to release or form bioactive component(s) is not specific for one microbial species. A range of ETEC strains was shown to be sensitive for the bioactive component, making this bioactivity of potential interest for application in animal husbandry.
Further research to elucidate the nature of the bioactive component in fermented leguminous seeds will be required.

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Chapter 5

First characterization of bioactive components in soya bean tempe that protect human and animal intestinal cells against enterotoxigenic *Escherichia coli* (ETEC) infection

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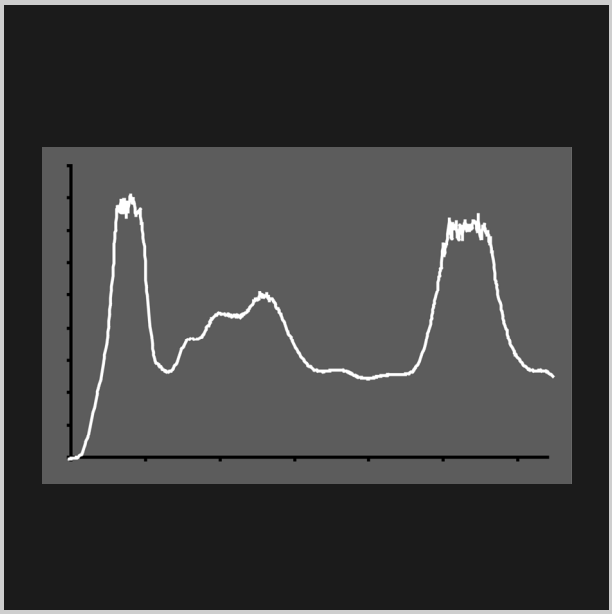

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Abstract

Tempe extracts can inhibit the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to intestinal cells and thereby can play a role in controlling ETEC-induced diarrhoea. The component responsible for this adhesion inhibition activity is still unknown. This research describes the purification and partial characterization of this bioactive component of tempe.

After heating, defatting and protease treatment, the extracts were found to remain active. However, after treatment with polysaccharide-degrading enzyme mixtures the bioactivity was lost. Ultrafiltration revealed the active component to be >30 kDa. Further purification of the bioactive tempe extracts yielded an active fraction with an increased carbohydrate content of higher arabinose content than the nonactive fractions. In conclusion, the bioactive component contains arabinose and originates from the arabinan or arabinogalactan side chain of the pectic cell wall polysaccharides of the soya beans, which is probably released or formed during fermentation by enzymatic modifications.

Introduction

Diarrhoeal diseases of humans and farm animals are frequently caused by infection with Enterotoxigenic *Escherichia coli* (ETEC). ETEC strains are associated with two major human clinical syndromes: weanling diarrhoea among children in developing countries and traveler's diarrhoea (Nataro and Kaper, 1998; Qadri *et al.*, 2005). ETEC is also an important and global cause of severe, watery diarrhoea in the offspring of some animal species such as newborn calves and suckling and weaned pigs (Nagy and Fekete, 2005).

Adhesion of ETEC to intestinal epithelium is known to be a prerequisite for colonization and infection of the intestinal tract. Adhesion of ETEC onto the small intestinal microvilli does not cause significant morphological changes, but it is a requirement for the secretion of enterotoxins that alter the behaviour of the enterocytes by increasing secretion and/or reducing absorption (Nagy and Fekete, 2005).

The adhesion of ETEC bacteria to intestinal epithelial cells can be mediated by (proteinaceous) fimbriae. These fimbriae bind to carbohydrate-specific receptors (binding sites) at the epithelial cell surface. Inhibition of adhesion can be due to carbohydrate epitopes that are structurally similar to the binding sites of the epithelial cells and, therefore, adhere to the bacteria and act by competitive inhibition. Nonadhering bacteria are subsequently removed by regular bowel-cleansing mechanisms (Ofek *et al.*, 2003; Sharon, 2006; Pieters, 2007). Human breast milk oligosaccharides are known to act as these so-called antiadhesins (Bode, 2006).

Other studies concluded that proteinaceous components released from lactic acid bacteria decrease the adhesion of ETEC to piglet ileal mucus (Blomberg *et al.*, 1993). Also, bifidobacteria are known to secrete a proteinaceous component that prevents the adhesion of ETEC to intestinal epithelial cell lines (Fujiwara *et al.*, 2001). This adhesion inhibition can be due to competitive exclusion between bacteria, steric hindrance, or chemical changes of adhesion receptors of the epithelial cells.

Tempe is a traditional, fermented soya bean product made from dehulled, soaked, and cooked soya beans inoculated with mould, usually a *Rhizopus* species. During the fermentation mycelial biomass is formed and the fungal enzymes breakdown the soya bean macromolecules. This contributes to the development of a desirable texture and flavour in the product. It also inactivates or eliminates some soya antinutritional components (Nout and Kiers, 2005).

Carbohydrases are used in piglet diets to improve nutrient utilization, and it has been reported that the addition of certain carbohydrases to piglet diets reduced the frequency

and severity of diarrhoea (Partridge, 2001). Other research showed that the hydrolysis products of nonstarch polysaccharides of soya bean meal are beneficial in fluid balance during ETEC infection (Kiarie *et al.*, 2008). The enzymatic activity during fermentation could give the same beneficial effects to the soya beans.
Earlier research indicated that the severity of diarrhoea in ETEC-induced weaned piglets was less when these piglets were fed on tempe instead of toasted soya beans (Kiers *et al.*, 2003). Kiers et al. (2007) investigated the effect of tempe on fluid absorption in piglets and suggested that a water-soluble, high molecular mass fraction (>5 kDa) of tempe is able to protect against fluid losses induced by ETEC. As a consequence, this fraction could thus play a role in controlling ETEC-induced diarrhoea (Kiers *et al.*, 2007). Furthermore, tempe extracts can inhibit the adhesion of ETEC to intestinal epithelial cells in both piglet brush border cells (Kiers *et al.*, 2002) and human epithelial (Caco-2) cells (Roubos-van den Hil *et al.*, 2009). Tempe extracts were found to interact with ETEC bacteria and could possibly form a layer coating (part of) the bacterial surface, which may contribute to the observed decrease of ETEC adhesion to intestinal epithelial cells (Roubos-van den Hil *et al.*, 2009).
In this research we describe the characterization of the bioactive components in tempe, which inhibit adhesion of ETEC to intestinal cells.

Material and methods

Materials

Dehulled yellow seeded soya beans (*Glycine max*) were supplied by Kleinjan VOF. (Rhoon, The Netherlands). Malt extract agar (MEA) and peptone were purchased from Oxoid (Basingstoke, UK). Brain-heart infusion broth (BHI) was purchased from Becton Dickinson (Sparks, MD, USA). Enzymes were obtained from various sources as described in table 1. All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

Tempe preparation

Dehulled, full-fat yellow-seeded soya beans were soaked overnight in tap water using three cycles of accelerated acidification at 30ºC (Nout *et al.*, 1987). Next, the beans were washed with tap water and cooked in fresh tap water for 20 min in a bean:water ratio of 1:3 (v/v). Subsequently, the beans were cooled and superficially dried at room temperature on perforated trays. For the fungal inoculation of the soya beans a sporangiospore suspension was used. The suspension was prepared by scraping off the

sporangia from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU573) grown on MEA for 7 days at 30ºC, and suspending them in sterile distilled water with 0.85% (w/v) NaCl and 0.1% (w/v) peptone (PPS). After inoculation of the cooked beans with the sporangiospore suspension (10 ml kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans), viable sporangiospores of *R. microsporus* were enumerated as colony-forming units (CFU) as described previously (Nout *et al.*, 1988). The beans (450 g) were packed into hard plastic, perforated boxes (205 x 90 x 45 mm) and incubated for 72 h. Fermented soya beans (tempe) were freeze-dried and milled (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) using a 0.5 mm sieve and stored at -20ºC until further processing.

Table 1: Overview of carbohydrases used.

Name	Abbreviation	Main substrate(s)	Source
Endo-β-1,4-galactanase[1,3]	GAL	Galactan	*Asp. niger*
Arabinofuranosidase A[2,3]	ARA-A	Arabinan	*Asp. niger*
Arabinofuranosidase B[2,3]	ARA-B	Arabinan	*Asp. niger*
α-Mannosidase[4]	α-MAN	Mannan	Jack beans
Endo-β-mannanase[5]	β-MAN	Mannan	*Asp. niger*
Gammanase[6]	GAM	Mannan	
Mix of GAL, ARA-A and ARA-B	GAL+ARA	(Arabino)galactan, arabinan	
Driselase[4]	DRI	Pectine, hemicellulose	Basidomycetes
Pectinex Ultra SP-L[6]	PEC	Pectine, hemicellulose	*Asp. aculeatus*
Rapidase Liq+[3]	LIQ	Pectine, hemicellulose	

[1] purified by Van de Vis *et al.* (1991)
[2] purified by Rombouts *et al.* (1988)
[3] Supplied by: DSM, Delft, The Netherlands
[4] Supplied by: Sigma, St. Louis, MO
[5] Supplied by: Megazyme, Bray, Wicklow, Ireland.
[6] Supplied by: Novozymes, Bagsvaerd, Denmark

Bioactivity assay

Brush borders were isolated from epithelial cells of the intestine of early weaned pigs as described before (Sellwood *et al.*, 1975). The concentration of brush border was between 10^5 and 10^6 brush border ml^{-1} in phosphate-buffered saline (pH 7.2) (PBS). The brush borders were tested for adhesion of ETEC K88 strain ID 1000 and *E. coli* (O149:K91) strain ID1084 (source, ID-Lelystad, Lelystad, The Netherlands). The

ETEC K88-positive (ID 1000) and K88-negatieve strains (ID1084) were grown overnight at 37ºC in BHI. The cultures were centrifuged (10 min, 3000 *g*, 20°C) and washed twice in PBS, followed by suspension of the pellets in PBS to an optical density of 0.75 correlating with a concentration of 10^9 CFU ml^{-1}.
Freeze-dried fractions of tempe extracts (10 mg) were suspended in 1 ml of PBS (1 w/v %), mixed for 1 h, and centrifuged (10 min, 10000 *g*, 20ºC). Dilutions of the supernatants (0.25 and 0.1 w/v %) were prepared in PBS. Aliquots (30 µl) of each of the three solutions were mixed with 30 µl of bacterial suspension and 30 µl of brush borders and incubated at room temperature with continuous gentle shaking for 1 h. The number of bacteria adhering to 12 brush borders, each derived from a single epithelial cell, was determined by phase contrast microscopy. The percentage adhesion was calculated as the average number of ETEC K88 per brush border relative to a PBS control.

Preparation of fraction "active 1"
The tempe extracts were characterized by measuring the bioactivity after different treatments, that is, defatting, protease treatment, heating, and filtration, had been applied. Defatting was performed by three consecutive additions of hexane (1:10 (w/v) tempe: hexane) followed by filtration through a folded paper filter (Schleicher & Schuell GmbH, Dassel, Germany) and air-drying, all at 20ºC. Tempe extracts were prepared by suspending 75 g of defatted tempe powder in 1 l of distilled water and stirring for 1 h at room temperature while the pH was adjusted to pH 8.0 with 2 M NaOH every 30 min. The soluble tempe material was obtained by collection of the supernatant after centrifugation (30 min, 25000 *g*, 20ºC). The supernatant was filtered through a folded paper filter followed by a second centrifugation step.
Heat treatment was done by heating the extract for 15 min at 100°C. For protease enzyme treatment, proteinase K or pronase E was added (0.01% w/v) and incubated overnight at room temperature, followed by heating for 10 min to inactivate the enzymes. Then, the extracts were filtered using centrifugal filters with a membrane cutoff of 30 kDa (Centriprep YM-30, Millipore, Billerica, MA, USA). Both filtrates and retentates were freeze-dried and used for the bioactivity assay. Also, combinations of treatments were performed (see figure 2 for combinations of treatments) and tested for bioactivity. Finally, the so-called fraction "active 1" was obtained by a combination of defatting, filtration, proteinase K treatment, and heating. Figure 1 shows the order of treatments to obtain fraction "active 1".

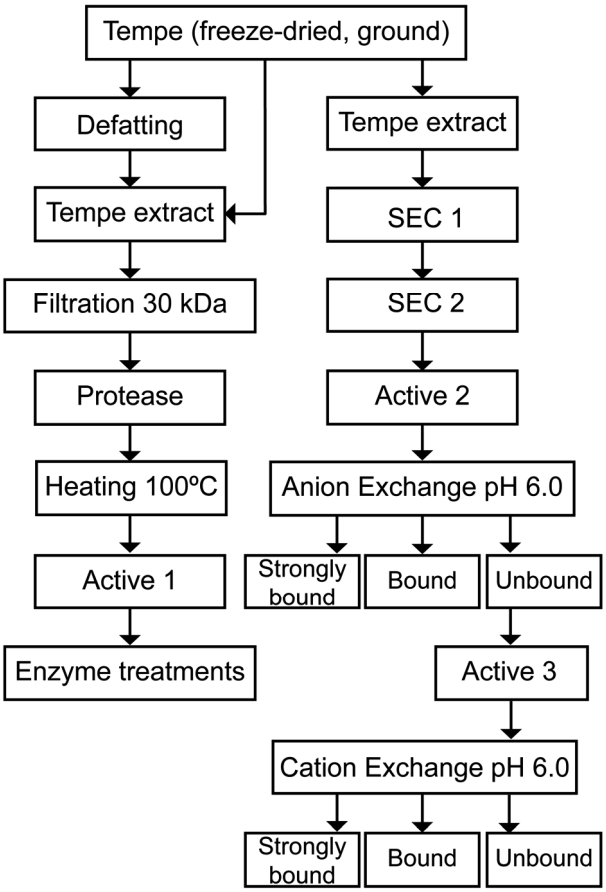

Figure 1: Schematic overview of the different treatments performed with the tempe extracts.

Enzyme treatments of fraction "active 1"

Freeze-dried fraction "active 1" (10 mg ml^{-1}) was solubilised in 20 mM NaOAc-buffer (pH 5.0). Powdered enzymes (10 mg ml^{-1}) were solubilised in NaOAc buffer (pH 5.0). Carbohydrases (table 1) were added (20 µl) each to 1 ml of fraction "active 1" solution and the mixture was incubated overnight at room temperature (20°C) followed by heating for 10 min at 100°C.

After enzyme incubations, the samples were tested for bioactivity, and carbohydrate molecular weight distributions were determined. The molecular weight distribution was determined by high-performance size exclusion chromatography (HPSEC) using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA). Freeze-dried fractions

were dissolved in 0.2 M sodium nitrate (5 mg ml^{-1}) and injected onto three TSK Gel columns in series (superAW 2500, superAW 3000, superAW 4000, each 6 mm × 150 mm, Tosohaas, Stuttgart, Germany) in combination with a superAW Lguard column (Tosohaas). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.6 ml min^{-1}. The eluate was monitored using a refractive index (RI) detector (RI61, Shodex, New York, NY, USA). The system was calibrated with pullulan standards (mass range from 180 to 4.04 x 10^5 kDa).

Next, the enzyme-treated samples were dialysed using 3 kDa filters (Microcon YM-3 3,000 NMWL, Millipore) to remove the degradation products. The monosaccharide composition of the remaining part was determined after hydrolysis using combined methanolysis/trifluoroacetic acid (TFA) hydrolysis. Sample solutions (100 µl; 1 mg ml^{-1}) were dried using a vacuum oven at 40ºC during 4 h. Next, 1.0 mL of 2 M HCl in dry methanol was added, flushed with nitrogen, and closed. The samples were incubated for 16 h at 80ºC, followed by cooling, and evaporated under a stream of dry air at 40ºC. Subsequently, 0.5 ml of 2 M TFA was added, and the samples were heat treated for 1 h at 121°C in screw-capped glass vials in a heating block, followed by evaporation under a stream of dry air at 40ºC. The remaining material was washed twice with methanol, followed by evaporation, and finally dissolved in 1 ml of distilled water and diluted 10 times.

The monosaccharide composition was determined by high-performance anion exchange chromatography (HPAEC) using a Dionex ICS 3000 system (Dionex) connected with a PAD detector (Dionex ICS3000). A CarboPac PA-1 column (2 x 250 mm, Dionex) was used in combination with a CarboPac PA guard column (2 x 50 mm, Dionex). Samples were injected (20 µl) onto the column and eluted for 30 min with Millipore water at a flow rate of 0.3 ml min^{-1}, followed by a 15 min linear gradient of 0-40% (v/v) 1 M NaOAc in 0.1 M NaOH, continued by 5 min of elution with 1 M NaOAc in 0.1 M NaOH, followed by 5 min of elution with 0.1 M NaOH. Finally, the column was equilibrated with Millipore water for 25 min. As the postcolumn mobile phase, 0.5 M NaOH was used, to allow a proper detection, with a flow rate of 0.1 ml min^{-1}. A standard was made with 1 mg ml^{-1} fucose, rhamnose, arabinose, xylose, galactose, glucose, mannose, glucuronic acid, and galacturonic acid and treated with the same procedure as the samples.

Preparation of fraction "active 2"

For isolation of the bioactive substance in tempe, freeze-dried tempe powder (200 g) was suspended in 2 l of 35 mM sodium phosphate buffer (pH 8.0) and stirred for 1 h at

room temperature (20°C), while the pH was adjusted every 30 min. The insoluble parts were removed by centrifugation (30 min, 25000 *g*, 20ºC) followed by filtration of the supernatant through a folded paper filter and a second centrifugation step.

Size exclusion chromatography was performed using an Akta explorer system (GE Healthcare, Uppsala, Sweden). The supernatant (200 ml) was applied onto a Superdex 200 PG column (4.5 l, fractionation range from 1 x 10^4 to 6 x 10^5 Da, GE Healthcare) and eluted with 35 mM sodium phosphate buffer (pH 8.0) at a flow rate of 40 ml min^{-1}. The eluate was monitored at 210 and 280 nm, and fractions (210 ml) were collected. Five runs were performed, and fractions eluting at the same time were pooled. Pooled fractions were dialysed against distilled water with a 12-14 kDa dialyzing tube (Medicell international, London, UK) and freeze-dried. Freeze-dried fractions were tested for bioactivity.

To purify the active part, the active freeze-dried fractions that eluted between 1.9 and 2.9 l were pooled and solubilised in 250 ml of Millipore water. The solution was again applied onto the Superdex 200 PG, and fractions (200 ml) were collected. The fractions were dialysed, freeze-dried, and tested for bioactivity. The active fractions were pooled and further described as fraction "active 2".

Fraction "active 2" (10 mg ml^{-1}) was dissolved in 35 mM sodium phosphate buffer (pH 6.0). Anion exchange was performed using a glass filter (diameter = 25 mm) filled with 3 ml of the resin (DEAE CL 6B, GE Healthcare) on top of a vacuum Erlenmeyer. First, 3 ml of 35 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl was eluted, to maximize exchangeable counterions, followed by washing of the resin with 15 ml 35 mM phosphate buffer (pH 6.0). Next, 3 ml of fraction "active 2" solution was added. The unbound fraction was collected, and 3.5 ml 35 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl was added to the resin and used to elute the bound material. Finally, 1M NaOH was added to clean the resin and remove the so-called strongly bound fraction, which was immediately adjusted to pH 6.0 with HCl. The unbound, bound, and strongly bound fractions were dialyzed, freeze-dried, weighed, and tested for bioactivity.

The unbound fraction (active 3) was again dissolved in 35 mM sodium phosphate and brought to pH 3.5 with formic acid and eluted through the cationic resin SP Sepharose Fast Forward (GE Healthcare) with the same solutions as described for the anion exchange resin with the exception of using a 35 mM sodium phosphate/formic acid buffer (pH 3.5) instead of the 35 mM sodium phosphate buffer (pH 6.0). The fractions collected were dialyzed, freeze-dried, weighed, and tested for bioactivity.

Results

Characterization of the active component in tempe

Different treatments were performed to characterize the bioactive components in tempe. Figure 2 shows the bioactivity of the untreated tempe extracts as well as the extracts after heating (HE), defatting (DF), proteinase K and Pronase E treatment (PROK and PROE), and ultrafiltration (<30 or >30).

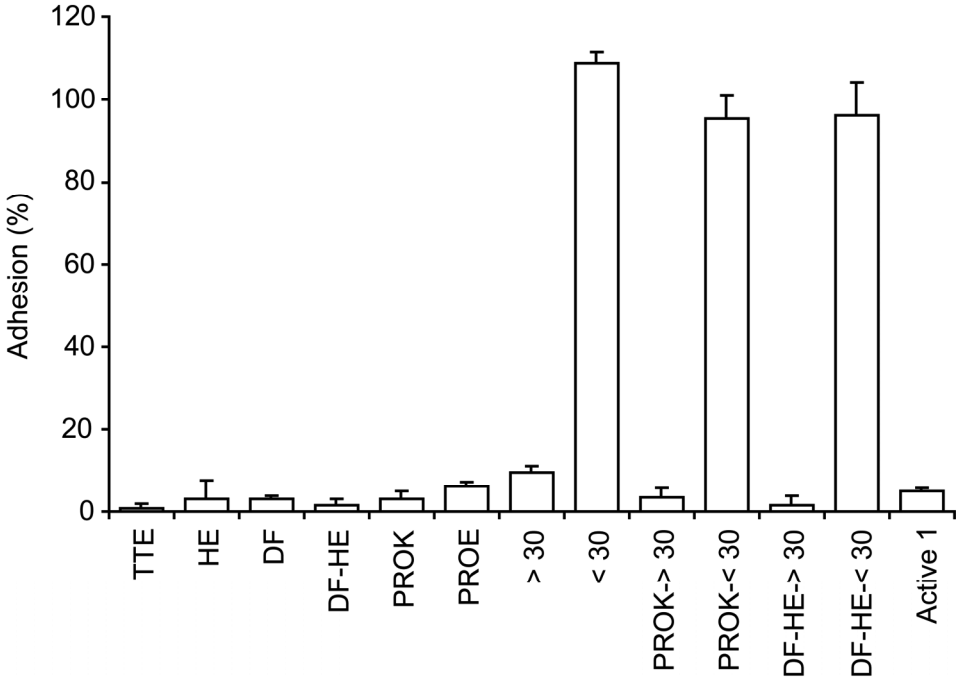

Figure 2: Bioactivity of tempe extracts after different treatments. Concentration of the extracts is 10 mg ml^{-1}. Bars represent average adhesion (n=12) expressed as percent adhesion compared to a control without any addition of tempe extracts. Error bars represent standard error of the mean. TTE, total tempe extract; HE, cooked tempe extract; DF, defatted tempe extract; PROK, tempe extract treated with proteinase K; PROE, tempe extract treated with pronase E; >30: tempe extract >30 kDA; <30: tempe extract <30 kDa; active 1, combination of DF, >30, PROK and HE.

The results show that the activity remained after defatting, protease treatment, and heating at 100ºC of the extracts. Filtering with a cutoff membrane of 30 kDa showed

that all activity was found in the retentate, which contained all material >30 kDa. Various treatments were combined, and bioactivity was present after the combination of defatting, protease treatment, heating at 100ºC and ultrafiltration and is presented as "active 1" in figure 2. The results on heating and protease treatment suggest that the bioactive component is not proteinaceous. The monosaccharide composition of the polysaccharides present in fraction "active 1" was determined. This fraction consists of 48% (w/w) of carbohydrates. The main monosaccharides of this fraction were arabinose, galactose, and galacturonic acid as shown in table 2.

Table 2: Monosaccharide composition (mol %) after degradation with Gammanase (GAM), Galactase + arabinofuranosidase (GAL+ARA), driselase (DRI) and pectinex Ultra SP (PEC) and dialysis.

	Fuc[1]	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA	Total sugars (%)[2]
Active 1	6.3	nd[3]	29.6	25.2	3.6	9.5	7.4	14.1	4.4	48.0
GAL+ARA	7.8	nd	29.0	12.2	3.3	13.3	10.1	17.0	7.2	28.8
DRI	8.0	nd	32.8	12.4	3.3	11.7	7.5	18.7	5.7	52.0
GAM	9.8	nd	5.3	15.3	5.1	16.7	12.3	27.1	8.5	33.3
PEC	11.4	nd	7.1	10.4	5.5	9.6	28.0	12.0	16.2	13.6

[1]Results are averages of 2 replicates. Fuc, fucose; Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; GalA, galacturonic acid; GlcA, glucuronic acid.
[2]Total sugar expressed as weight % of the total fraction.
[3]nd, not determined.

Next, fraction "active 1" was treated with various carbohydrate-degrading enzymes (listed in table 1), which were selected on the basis of the sugar composition (table 2), and the remaining bioactivity was measured. The results are shown in figure 3. After treatment with gammanase (GAM), Pectinex Ultra SP (PEC), and Liq+ (LIQ), the bioactivity was lost. The other enzyme-treated extracts remained bioactive. The enzymatic degradation of the tempe extracts was then evaluated with HPSEC. Figure 4 shows the chromatograms of fraction "active 1" (gray line) and the fractions "active 1" after enzymatic degradation with the enzymes (black lines). The chromatograms of arabinofuranosidase A (ARA-A) and arabinofuranosidase B (ARA-B) are not shown because almost no degradation was observed. The chromatograms show different

degradation profiles caused by the various enzymes. The chromatograms of GAM, PEC and LIQ (which caused loss of bioactivity) showed a clear degradation, particularly of material eluted at low elution volumes (between 8 and 10 min) in the chromatogram (size 65-796 kDa).

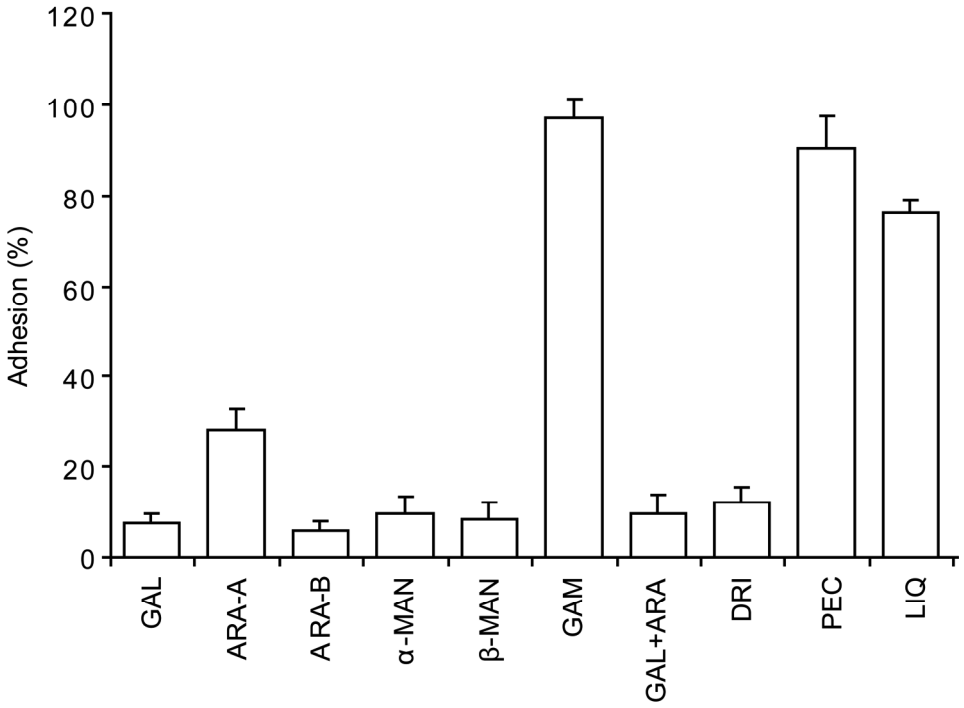

Figure 3: Bioactivity of fraction "active 1" after treatment with various enzymes. Concentration of the extracts is 10 mg ml^{-1}. Bars represent average adhesion (n=12) expressed as percent adhesion compared to a control without any addition of tempe extracts. Error bars represent standard error of the mean. GAL, Endo-β-1,4-galactanase; ARA-A, Arabinofuranosidase A; ARA-B, arabinofuranosidase B; α-MAN, α-Mannosidase; β-MAN, Endo-β-mannanase; GAM, Gammanase; GAL+ARA, Endo-β-1,4-galactanase and Arabino-furanosidase A and B; DRI, Driselase; PEC, Pectinex Ultra SP-L; LIQ, Rapidase Liq+.

Two bioactive samples (treated with endogalactanase with arabinofuranosidase A and B (GAL+ARA) and Driselase (DRI)) and two nonactive samples (GAM and PEC) were selected and dialyzed. During dialyses the material <3 kDa, which eluted around 12.5 min, was removed. From the remaining material the sugar composition was

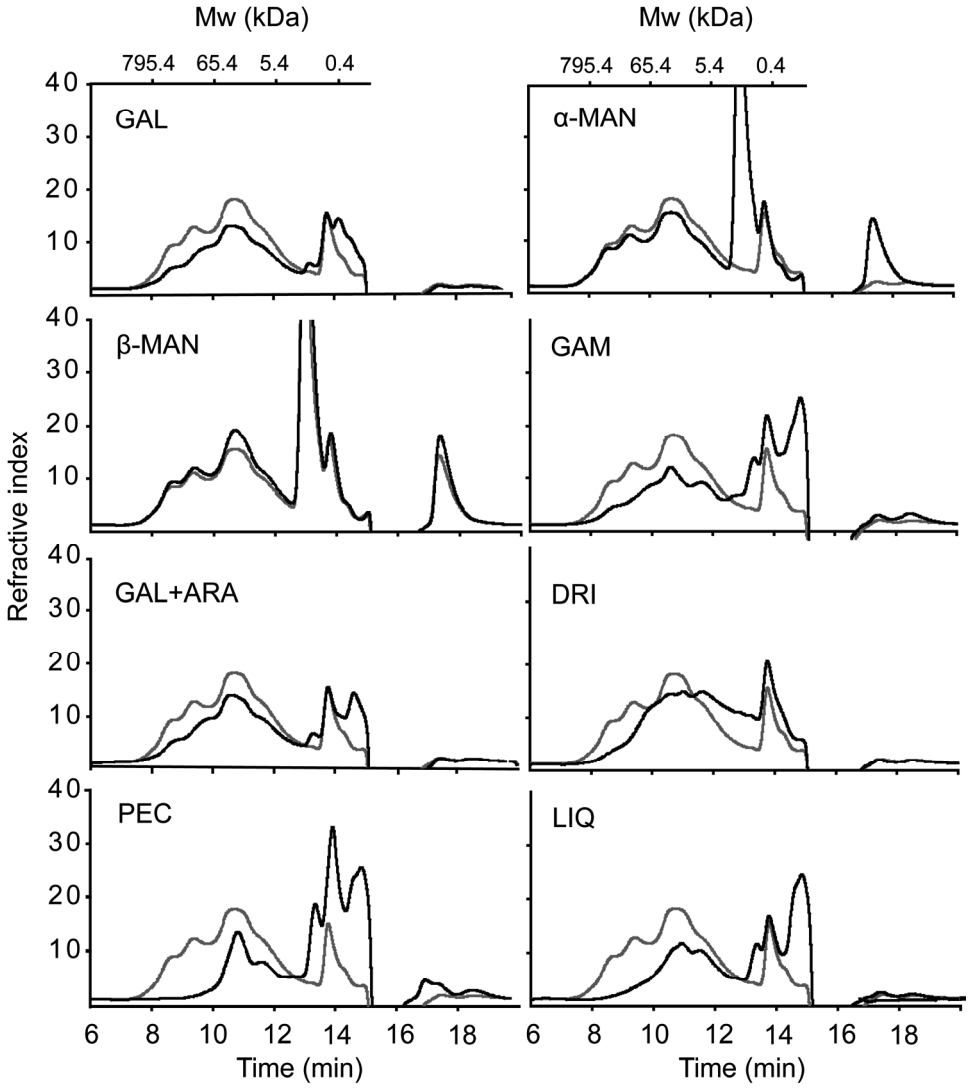

Figure 4: High-performance size exclusion chromatograms of tempe extract (gray lines) and tempe treated with various enzymes (black lines). GAL, Endo-β-1,4-galactanase; α-MAN, α-Mannosidase; β-MAN, Endo-β-mannanase; GAM, Gammanase; GAL+ARA, Endo-β-1,4-galactanase and Arabinofuranosidase A and B; DRI, Driselase; PEC, Pectinex Ultra SP-L; LIQ, Rapidase Liq+.

determined (table 2). After treatment with GAM, GAL+ARA and PEC, a decrease in total sugars was observed (data not shown), which was expected because of the removal of the mono- and oligosaccharides released during dialysis. After incubation with DRI, the polysaccharides were not degraded to monosaccharides, but to intermediate sized molecules as shown in the chromatogram (figure 4).
After degradation with GAM and PEC, the fraction “active 1” lost activity and showed a decrease in arabinose residues from 29.6 to 5.3 and 7.1 mol %, respectively, whereas galactose decreased in all enzyme-treated samples (table 2). Despite the reduction in galactose, the GAL+ARA and DRI degraded samples remained bioactive. These two samples showed much higher molar proportions of arabinose than the GAM and PEC degraded samples. The molar proportion of galacturonic acid did not show any relationship with the decreased activity caused by GAM and PEC. Also, the recovery of individual sugars after enzyme treatment was calculated. The GAM and PEC treatment resulted in a removal of 88 and 93% (w/w) of all arabinose residues present in the extract before the enzyme treatment, respectively (no further data shown).

Purification of the active component

The tempe extract was purified (figure 1) using two steps of size exclusion chromatography, labeled SEC 1 and SEC 2. Figure 5A shows the SEC elution pattern of tempe extract. In this figure the fractions collected and their corresponding activities are also indicated. The bioactivity was measured for the indicated fractions 1-11, because the active part is >30 kDa.
The activity was situated mainly in the intermediate part (fractions 4-6) of the chromatogram. At all three concentrations applied the adhesion was completely inhibited. The activities found are comparable to the activity of the total tempe extract (TTE) applied at 1% (w/v), and these fractions even had a stronger adhesion inhibition capacity at the lower concentrations. This concentration effect was also observed in the fractions that are eluted before or just after the most active fractions. The material of all runs eluting between 1.9 and 2.9 l (intermediate peaks) was pooled and again applied onto the SEC column. The resulting elution pattern with the fractions collected and corresponding bioactivities is shown in figure 5B. Fractions 4-6 had the highest activities. They were located just before the main peak in the chromatogram; thereafter, the inhibition activity decreased quickly.
After SEC 2, the active material eluted between 1.8 and 2.4 l (fractions 4-6) was pooled and denoted as fraction “active 2”. This material was eluted through an anion exchange resin column. Unbound, bound and strongly bound fractions were collected,

representing 38, 13 and 29% (w/w) of the starting material, respectively. The bioactivity was measured, and fraction "active 2" showed an adhesion of 11.1 ± 3.6% (average ± SEM) at a concentration of 0.1% (w/v) extract.

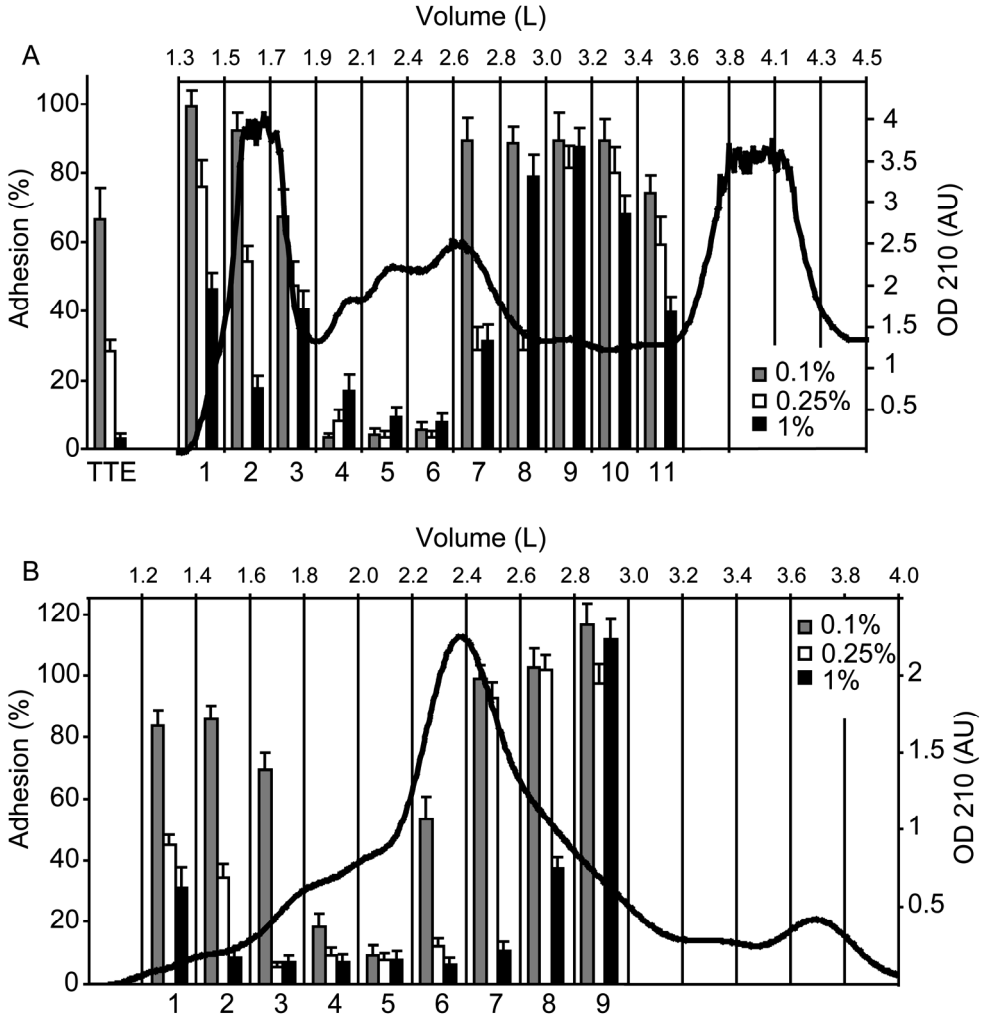

Figure 5: Size exclusion elution patterns of SEC 1 and SEC 2 with corresponding bioactivities (A) SEC 1; (B) SEC 2. TTE, total tempe extract, The lower X-axis represents the fraction numbers; the upper X-as represents the elution volume. Bioactivity is measured in three concentrations 0.1, 0.25 and 1% (w/v). Bars represent average adhesion (n=12) expressed as percent adhesion compared to a control without any addition of tempe extracts. Error bars represent the standard error of the mean.

The unbound material showed an adhesion of 14.0 ± 3.8% (average ± SEM), and the bound and strongly bound fraction 70.0 ± 4.1% and 99.3 ± 7.5, respectively. Obviously, the bioactive material was present in the unbound material, and this part is called "active 3". Subsequently, the fraction "active 3" was applied onto a cation exchange column. Again, unbound, bound, and strongly bound fractions were collected, representing 14, 38, and 32% (w/w) of the material loaded onto the column, respectively. The bioactivity was measured, and the highest inhibition activity was found in the unbound material, 36.0 ± 5.2% (average ± SEM) compared with the bound and strongly bound materials, 84.0 ± 11.5 and 74.0 ± 7.2%, respectively, all at a concentration of 0.1% (w/v) extract. Because, part of the bioactivity was lost by the cationic exchange, we continued our experiments with the bioactive fraction "active 3".

Table 3: Monosaccharide composition of fraction "active 3" (mol %).

	Fuc[1]	Rha	Ara	Gal	Glc	Ma[1]	Xyl	GalA	GlcA	Total sugars (%)[2]
Active 3	1.5	nd[3]	50.7	19.0	7.4	21.0	nd	0.2	0.2	56.4

[1]Results are averages of 2 replicates. Fuc, fucose; Rha, rhamnose; Ara, arabinose; Gal,galactose; Glc, glucose; Man, mannose; Xyl, xylose; GalA, galacturonic acid; GlcA, glucuronic acid.
[2]Total sugar expressed as weight % of the total fraction.
[3]nd, not determined

Monosaccharide analysis was performed on fraction "active 3" (table 3). This fraction contained 59% (w/w) sugars, which is higher than found in fraction "active 1" (table 2). Also, it exhibited a higher molar proportion (50%) of arabinose than fraction "active 1". The ratio between arabinose and galactose moieties had changed from 1.2:1 to 2.7:1 for active 1 and active 3, respectively.

Discussion

We examined the chemical component(s) that protect intestinal cells from adhesion of ETEC bacteria. The bioactivity was tested on piglet intestinal brush border cells, but it is also active in inhibiting adhesion to human cells (Roubos-van den Hil *et al.*, 2009). The fractions of the first size exclusion chromatographic separation (SEC 1) experiment were also tested for their inhibition capacities to human Caco-2 intestinal

cells. Results showed activity in fractions 5 and 6 with an inhibition of the adhesion of ETEC K88 to the intestinal epithelial cells of 60% (no further data shown). Earlier research found an inhibition of adhesion to Caco-2 cells by total tempe extracts of almost 50%, which may possibly reduce ETEC-induced diarrhoea in humans (Roubos-van den Hil *et al.*, 2009).

Because our earlier research (Kiers *et al.*, 2003; Kiers *et al.*, 2007) had shown that the protective effect of tempe against ETEC-induced diarrhoea was not diminished by *in vitro* or *in vivo* digestion, we used nondigested tempe extracts in this study for reasons of convenience and to avoid purification difficulties.

After ultafiltration of the tempe extracts, the bioactive component was recovered in the >30 kDa fraction. Also, the SEC 1 and SEC 2 experiments showed that the bioactive component is intermediate in size. In the research of Kiers *et al*. (2003) the bioactive component was recovered in the >5 kDa fraction. They found that high molecular mass (>5 kDa) predigested and undigested tempe extracts showed equal protection against ETEC-induced fluid loss. The HPSEC patterns of these predigested and undigested tempe extracts were also identical in the high molecular weight part (Kiers *et al.*, 2003).

Tempe contains a diversity of microbial enzyme activities. Proteolytic enzyme activity in the gut could degrade intestinal receptors for ETEC as was shown before for bromelain (Chandler and Mynott, 1998). After heating of the tempe extracts, the proteolytic enzymes were inactivated (data not shown). Nevertheless, the tempe extracts were still bioactive after heating at 100ºC (figure 2); thus, we concluded that the proteolytic enzyme activity was not responsible for the bioactivity.

Furthermore, it was shown that the bioactive component was not removed by defatting the tempe extract and the bioactivity was not influenced by two broad spectrum proteolytic enzymes. Although the fraction “active 1” did not contain carbohydrates only, these findings make it unlikely that the active component was either proteinaceous or lipidic.

Carbohydrates have been described to prevent infection with bacterial pathogens by competitive inhibition (Sharon, 2006). More specifically, soy polysaccharides have also been shown to reduce the duration of diarrhoea in children (Brown *et al.*, 1993). Previous research on tempe adhesion inhibition indicated that inhibition of adhesion by tempe extracts is caused by an interaction between the ETEC bacteria and the tempe extracts (Roubos-van den Hil *et al.*, 2009). The interaction could be caused by adhesion between the fimbriae of the ETEC and carbohydrate epitopes that are

structurally similar to the binding sites on the epithelial cells. This strengthens the hypothesis that the bioactive component is made up of carbohydrates.

Tempe contains cell wall polysaccharides from different origins: (1) from soya beans and (2) from the mould *R. microsporus*. Furthermore, tempe is rich in lactic acid bacteria. The bioactivity of the tempe extracts is enhanced during fermentation (Roubos-van den Hil *et al.*, 2009). This can be due to the growth of the mould or lactic acid bacteria or the degradation of macromolecules during fermentation.

Previously, we investigated (Roubos-van den Hil *et al.*, 2010) the bioactivity of tempe extracts produced with various substrates and the same starter pure culture. All leguminous fermented substrates were bioactive, whereas cereal-derived tempe products (which allowed a similar mycelial growth of *Rhizopus*) showed no bioactivity at all. Also, after inoculation of soya beans with lactic acid bacteria, bioactivity was not induced (Roubos-van den Hil *et al.*, 2010). These results suggest that the active component is released or formed by enzymatic breakdown from leguminous substrates.

Carbohydrates in soya beans contain mainly cell wall polysaccharides and the small sugars fructose, raffinose, and stachyose. These small sugars were removed during soaking, cooking, and partially by fermentation of the soya beans and the remaining were removed during filtration of the processing of fraction “active 1”. The cell wall polysaccharides of soya are (partly) degraded during fermentation by the enzymes of the mould, which leads to enhanced solubility (Kiers *et al.*, 2000). The major carbohydrases of *Rhizopus. oligosporus* grown on tempe include polygalacturonases, endocellulases, xylanases, and arabinanases (Sarrette *et al.*, 1992). During fermentation the arabinogalactan and pectin fractions are predominantly solubilised (De Reu *et al.*, 1997). The major monosaccharide constituents in soya cell walls are galactose, glucose (from cellulose), arabinose, and galacturonic acid (Huisman *et al.*, 1998). Fraction “active 1” is rich in arabinose, galactose and galacturonic acid, which corresponds to the observation (De Reu *et al.*, 1997) of degradation or solubilisation of pectin and arabinogalactan during fermentation.

The monosaccharide composition of the remaining polysaccharides in fraction “active 1” after enzyme treatment showed that arabinose is an important component of the bioactive fractions. Enzymes that were able to degrade the arabinose-containing polysaccharides were all responsible for a loss of bioactivity, whereas the enzymic removal of galactose and galacturonic acid from the polysaccharides did not specifically cause a loss of activity.

After further purification of the active component with SEC and anion exchange (fraction active 3), an increase in molar proportion of the arabinose in the active

fraction was also observed, which strengthens this statement. Research published elsewhere (Ofek *et al.*, 2003; Bode, 2006; Sharon, 2006) indicated that sugars other than arabinose may inhibit bacterial adhesion by interaction with the bacteria.
Arabinose, in addition to galactose, is an important monosaccharide in pectic cell wall polysaccharides of soya beans. Arabinose is especially present in the side chains of rhamnogalacturonans. Rather long arabinan side chains with degrees of polymerisation up to 30-35, in addition to the (arabino)galactan side chains, have been reported (Huisman, 2000). It is assumed that some structural epitopes of such arabinans or arabinogalactans are responsible for the bioactivity in tempe. During fermentation these structures become more soluble and are thereby more accessible to the intestinal cells.
Some commercial carbohydrates (mannose, arabinose, galactose, arabinogalactan, arabinan), were tested, but they were not bioactive (no further data shown).
Because the bioactivity is found only in fractions >30 kDa and is lost after incubation with some enzyme mixtures only a partial degradation of cell wall polysaccharides is needed to obtain the required structure needed for adhesion to ETEC in competition with the binding sites of the epithelial cells.
In conclusion, it is likely that the active component is of carbohydrate nature, containing arabinose. The bioactive component originates from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of the soya beans and is released or formed during fermentation by enzymatic modifications. Further research to fully characterize the chemical structure of the bioactive component is needed. A full characterization could open new possibilities for producing the bioactive component, probably via the addition of specific enzymes liberating the arabinose-containing medium-weight polysaccharides, which makes it a potential ingredient in food or feed matrices.

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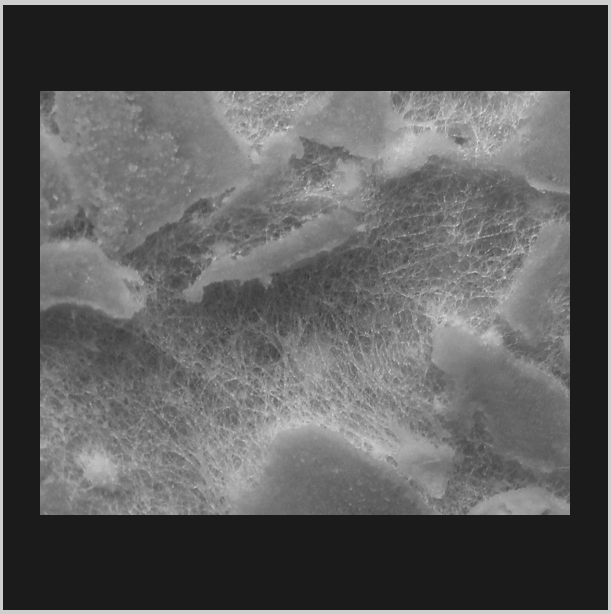
Chapter 6

General discussion



Introduction

Fermented soya bean foods are attractive products and enjoy an increasing interest because of their nutritional and health benefits. During the fermentation processes, enzymes that are synthesized by the microorganisms are important, since they carry out reactions, that contribute to the development of specific textures, tastes and aromas.

This research was performed with tempe, a *Rhizopus* fermented soya food. Nutritional and health aspects of tempe, have attracted scientific attention. Kiers (2001) observed that during the fermentation of soya beans a major degradation of macronutrients resulted in increased nutrient availability for pigs. This was shown by higher absorption of nutrients and better weight gain in early weaned piglets, fed with tempe, compared with soya beans. Moreover, tempe was found to reduce the incidence and severity of enterotoxigenic *Escherichia coli* (ETEC) induced diarrhoea in these early weaned piglets, compared to the usually fed toasted soya beans (Kiers *et al.*, 2003). *In vitro* research showed a strong inhibition of the adhesion of ETEC to intestinal cells, which is an essential step in infection (Kiers *et al.*, 2002). The mechanism to explain this protective effect is unknown and the molecular characteristics of the functional agent(s) required investigation.

The research in this thesis was performed to investigate in more detail the effect of tempe (components) on diarrhoea-associated bacteria. The focus of attention was to understand the mechanism of action and to test the specificity of the anti-diarrhoeal effect. Moreover, the chemical properties and composition of the bioactive component(s) were investigated.

The bioactive effects of tempe on bacteria

Since tempe was found to reduce the incidence and severity of ETEC induced diarrhoea, the effect of tempe on ETEC, and on other bacteria was investigated. Firstly, the antibacterial activity of tempe extracts towards a range of bacterial strains was investigated, as shown in table 1. A distinct antibacterial effect was observed against *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, whereas growth of *Lactobacillus plantarum*, isolated from tempe, was not affected. Also, *Bacillus* strains were inhibited by the tempe extract and a low antibacterial activity was observed against *Listeria* strains. This antibacterial effect towards *Listeria* was stronger when higher concentrations of tempe extract were used (data not shown). No antibacterial activity of tempe against *E. coli* or *Salmonella* was observed. Tempe extracts appear to be antibacterial mainly to certain Gram-positive bacteria. Other studies have also

shown antibacterial activity by tempe or a mould extract against Gram-positive bacteria (Wang *et al.*, 1969; Wang *et al.*, 1972; Kobayasi *et al.*, 1992; Kiers *et al.*, 2002).

Table 1: Antibacterial activity expressed as growth delay (h) of various strains exposed to tempe extracts (1% w/v)[2] compared to growth in BHI control.

Strain	Growth delay (h)
Listeria innocua	1.6
Listeria monocytogenes	1.0
Lactobacillus bulgaricus	>22[1]
Streptococcus thermophilus	>22
Lactobacillus plantarum (LU 857)	-2.0
Lactobacillus plantarum (LU 852)	-2.3
Escherichia coli K88 (ID 1000)	-0.3
Salmonella enteritidis (97-198)	0.0
Bacillus cereus (ATCC 14579)	6.3
Bacillus subtilis	>12.5

[1] >Means that the growth delay is longer than the total measurement period.
[2] Tempe extract (1% w/v) was prepared by solubilizing 10 g l^{-1} dry soluble material in BHI.

The research on the antibacterial effect towards *Bacillus* was described in chapter 3. During the fermentation of soya beans with *Rhizopus*, components are released with high antibacterial activity against *B. cereus* cells and spores. Optimum conditions for maximum antibacterial effect were established and the antibacterial spectrum to several *Bacillus* strains was determined. All *Bacillus* strains tested were strongly affected by the antibacterial action as expressed by growth delay compared to growth in control media without tempe. Vegetative cells of *Bacillus* were inactivated during the first 15 minutes of exposure to tempe extracts. After several hours a re-growth of bacteria was observed, which could be due to either a degradation or shortage of the antibacterial component or to a surviving sub-population. *B. cereus* spores were inactivated immediately after germination as shown by optical density and microscopic observations. This inactivation of the germinated spores appears to be caused by permeabilization of the cytoplastic membrane as shown by fluorescence microscopy.
Recently, the antibacterial effects of the well-known antibacterial component nisin against *Bacillus anthracis* spores were investigated (Gut *et al.*, 2008). While the results of that study also pointed to disruption of the membrane integrity, the tempe antibacterial component is nevertheless different from nisin, based on the sensitivity of nisin to heat and low pH, and its inactivation by different proteases. Furthermore, the

antibacterial activity was not found in pure *Rhizopus* strains, cultured on agar plates. This suggests that the antibacterial component(s) are degradation products of soya proteins, and that the fermentation starter microorganisms play a mediating role. The observed antibacterial component(s) can possibly play a role in food preservation and pathogen control.
The research of the anti-diarrheal effect of tempe, as investigated by Kiers *et al*. (2003), was continued. Since tempe extracts showed no antibacterial activity to ETEC bacteria, another explanation for the anti-diarrhoeal effect had to be found. Experiments had shown that tempe inhibits the adhesion of ETEC to piglet intestinal epithelial cells (Kiers *et al.*, 2002) and this was studied further as described in chapter 2. It revealed that adhesion inhibition to piglet brush border cells was caused by components present in aqueous extracts of all stages of tempe production. The highest activity was observed in the fermented products irrespective of the duration of fermentation. The extract of fermented soya beans also showed *in vitro* inhibition of adhesion to human intestinal epithelial Caco-2 cells.

The influence of tempe on the adhesion of ETEC to intestinal cells
An important step in the infection by ETEC bacteria is the adhesion to and colonization of the intestinal epithelial cells. If this adhesion could be disrupted by food products, the bacteria would not be able to colonize and could pass the intestine without causing infection (Nataro and Kaper, 1998; Nagy and Fekete, 2005).
The adhesion of ETEC bacteria to intestinal epithelial cells can be mediated specifically by fimbriae. These (proteinaceous) structures bind to receptors (binding sites) at the intestinal epithelial cell surface. Competition of this adhesion can be mediated by carbohydrates and proteins as depicted in figure 1. Carbohydrates, which are structurally similar to the binding sites of the epithelial cells, can adhere to the bacteria (Ofek *et al.*, 2003; Sharon, 2006; Pieters, 2007). For example, human breast milk contains many oligosaccharides that act as “anti-adhesins” (Bode, 2006). Also proteinaceous component(s) can interact with this specific adhesion by binding to the receptors at the intestinal epithelial cells. Blomberg *et al*. (1993) showed that proteinaceous components released from lactic acid bacteria could decrease the adhesion of ETEC to piglet ileal mucus. Bifidobacteria are also known to excrete a proteinaceous component that prevents the adhesion of ETEC to intestinal epithelial cells (Fujiwara *et al.*, 2001). Moreover, bacteria living in the mucus layer can prevent the attachment of pathogenic microbes by occupying available binding sites (Pluske *et al.*, 2002).

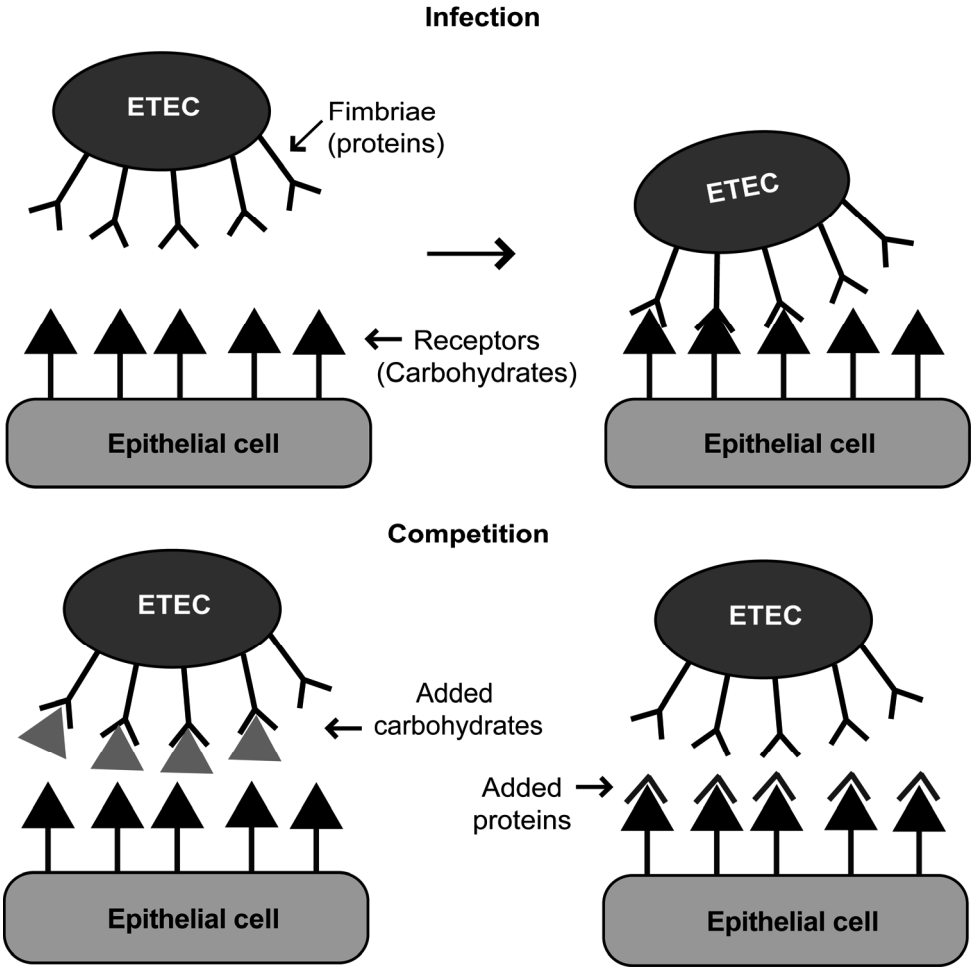


Figure 1: Infection of intestinal tissue cells by ETEC bacteria and the mechanisms of inhibition of adhesion by specific food components.

Another mechanism which has been shown to inhibit adhesion of ETEC to intestinal cells is by modification of the fimbriae with proteolytic enzymes. It was shown that the adhesion of ETEC was inhibited, possibly by degradation of the proteineous fimbriae. For example, bromelain, a proteolytic enzyme from pineapple stems, reduced ETEC adhesion to intestinal cells significantly (Chandler and Mynott, 1998). In this thesis it was shown that after heating, during which the proteolytic enzymes were inactivated,

the tempe extracts remain bioactive (chapter 5). Thus, proteolytic enzymes could not have been responsible for the adhesion inhibition.
In chapter 2, interactions between intestinal epithelial cells, ETEC and tempe extracts were measured. Pre-treatment of ETEC with tempe extracts resulted in strongly reduced adhesion. Washing of the bacteria, which removed non-bound tempe extract did not restore the adhesion, which suggested a strong interaction between ETEC and component(s) in the tempe extracts, which was not lost by washing steps.
Incubation of intestinal epithelial cells with tempe extracts prior to the ETEC addition reduced adhesion strongly. However, this reduced adhesion was restored by washing (removal of tempe) the epithelial cells before ETEC addition. These results point towards the interaction between ETEC and the tempe extracts as the mechanism of adhesion-inhibition. In addition, an adhesion of ETEC to tempe extracts was measured with tempe extracts bound to high binding polystyrene plates. More adhesion of ETEC bacteria was shown on wells coated with tempe extracts, which also provides evidence for the adhesion between ETEC and tempe extracts. Thus, the inhibition of adhesion is mediated by an interaction with the bacteria, which suggests the bioactive component to contain carbohydrates (figure 1).

Chemical characteristics of the bioactive component
In chapter 4, the effect of various fermentation substrates on the inhibition of ETEC adhesion to intestinal epithelial cells was described. During fermentation of various legumes (soya bean, cowpea, green pea and red bean), the bioactivity increased to a similar extent as was measured in fermented soya beans. Interestingly, the cereal (wheat, oat and barley) derived tempe products showed no bioactivity at all. The mould mycelial biomass itself is not responsible for the bioactivity, since the mould mycelia showed equally good growth in the cereal- and legume-derived tempe's. It was concluded that the bioactive component is specific for legumes and is released or formed by enzymatic breakdown during fermentation.
Tempe is traditionally fermented with moulds, mainly *Rhizopus* spp., but tempe also contains bacteria, i.e., lactic acid bacteria (LAB), *Bacillus* spp., and yeasts (Samson *et al.*, 1987; Nout and Rombouts, 1990), of which less is known in relation to their functions in tempe. Soya beans were fermented with several pure cultures of microorganisms, isolated from tempe or other fermented products. *Bacillus* spp., moulds and some yeasts were able to degrade macronutrients of the soya beans and showed bioactivity by inhibiting the adhesion of ETEC to intestinal cells. However, LAB fermentation of soya beans only resulted in lactic acid formation but not in

degradation of soya macromolecules, and did not result in bioactivity. Thus, the capability to release or form bioactive components from the soya beans is not specific for *Rhizopus* spp. Several strains that can degrade macromolecules could be used to elicit bioactivity in legumes.

The adhesion inhibition activity in fermented soya beans was described in chapter 2. It was observed that longer fermentation times (beyond 48 h) did not change the inhibitory activities. So, the bioactivity of the fermented soya bean extracts was formed within the first 48 h of fermentation.

The further characterization of the bioactive component present in fermented soya beans was described in chapter 5. Tempe extracts were defatted whereupon the extracts remained bioactive. After ultrafiltration the bioactive component was recovered in the >30 kDa part and size exclusion experiments showed the bioactive component to be intermediate in size. This is in agreement with Kiers *et al*. (2003), who found the bioactive component to be larger than 5 kDa. Furthermore, the bioactivity of the tempe extracts was not influenced by two broad spectrum proteolytic enzymes and heating at 100ºC. These results, combined with those of chapter 2, indicating that the inhibition of adhesion is caused by an interaction between ETEC and tempe extracts, make it likely that the bioactive component in tempe is of carbohydrate nature (figure 1).

Tempe contains cell wall polysaccharides from both fungal and soya bean origin. Since bioactivity is not caused by the biomass of the mould, we focused on the cell wall polysaccharides of soya. These cell wall polysaccharides are (partly) degraded during fermentation by the enzymes of the mould, which leads to enhanced solubility (Kiers *et al.*, 2000). Pectin and its arabinogalactan chains are predominantly solubilized during fermentation (De Reu *et al.*, 1997). Since all bioactivity is measured in water-soluble extracts, this increase of solubility could also be responsible for the higher bioactivity of the fermented extracts (enriched in these carbohydrates) compared with normal soya bean soluble extracts.

Galactose, glucose, arabinose and galacturonic acid are the major monosaccharide constituents in soya cell walls (Huisman *et al.*, 1998). After defatting, ultrafiltration, protease treatment and heating, the bioactive tempe extract was found to be rich in arabinose, galactose and galacturonic acid, which corresponds to the observation of the predominant degradation of pectin and arabinogalactan during fermentation of soya beans (De Reu *et al.*, 1997). The bioactive tempe extract was treated with different polysaccharide degrading enzymes, to obtain more detailed information about the bioactive component. Thereafter the monosaccharide composition was determined and arabinose was shown to be an important component of the bioactive fraction. After

enzymatic degradation of the arabinose containing polysaccharides, the bioactivity was lost, whereas the enzymatic removal of galactose and galacturonic acid from the polysaccharides did not specifically cause a loss of activity. Further purification experiments also showed an increase of arabinose in molar proportion in the purified active fractions.
The monosaccharide composition of leguminous and cereal grains, which were studied in chapter 4, was determined (table 1). These results show a higher molar proportion of arabinose in the leguminous materials compared to the cereal grains. This is in agreement with the bioactivity, which was only present in the leguminous materials. These results all strengthen the statement that the bioactive component contains at least arabinose.

Table 1: Monosaccharide composition (mol %) of leguminous and cereal grains fermented with *Rhizopus microsporus* (LU 573) after hydrolysis.

	Fuc[1]	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA
Soya	2.3	1.8	19.5	24.4	32.5	4.1	4.6	9.6	1.4
Organic Soya	3.1	1.6	31.8	23.3	21.0	4.9	5.9	7.4	1.0
Cowpea	nd	nd	30.0	14.4	40.5	2.4	7.7	4.6	0.4
Green pea	nd	0.5	35.5	10.2	42.4	1.4	3.6	6.1	0.3
Red bean	nd	1.0	22.3	23.5	44.4	0.5	3.0	5.0	0.5
Wheat	nd	nd	9.3	4.1	68.4	1.1	16.1	0.9	0.2
Oat	nd	nd	3.5	2.5	86.3	1.2	5.5	0.7	0.3
Barley	nd	nd	2.6	0.6	88.2	1.2	7.0	0.2	0.2

nd, not determined
[1] Fuc (fucose), Rha (rhamnose), Ara (arabinose), Gal (galactose), Glc (glucose), Man (mannose), Xyl (xylose), GalA (galacturonic acid), GlcA (glucuronic acid).

Arabinose is an important constituent of pectic cell wall polysaccharides of soya beans. Arabinose is especially present in the rather long arabinan and (arabino)galactan side chains of rhamno-galacturonans (Huisman, 2000). It is assumed that some structural epitopes of such arabinans or arabinogalactans are responsible for the bioactivity in tempe. During fermentation these structures become more soluble and are thereby more accessible to the intestinal cells.
The bioactivity was found only in leguminous substrates and increased during fermentation as a degradation product of the enzyme activity. Fractions containing

material >30 kDa are the most active, which suggests that only a partial degradation of cell wall polysaccharides is needed to obtain the bioactive component.
During fermentation the macromolecules are degraded by the enzymes of the microorganism (Nout and Kiers, 2005), but macromolecules can also be degraded by addition of enzymes, which possibly can give the same health effects. Carbohydrases could offer potential for the use in piglet diets to improve nutrient utilization and disease prevention, especially for the degradation of non starch polysaccharides (NSP) of the cell walls of animal feeds. After degradation of NSP by carbohydrases, the hydrolysis products may influence enteric bacterial infections in piglets (Pluske *et al.*, 2002; Meng *et al.*, 2005; Kiarie *et al.*, 2007). Indeed it was previously reported that the addition of carbohydrases to piglet diets reduced the frequency and severity of non-specific diarrhoea (Partridge, 2001). Kiarie *et al.,* (2008) investigated the effect of NSP hydrolysis products of soya bean meal against ETEC infected piglets *in situ* with intestinal segments. It was shown that NSP hydrolysis products of soya bean meal were beneficial in maintaining fluid balance during ETEC infection (Kiarie *et al.*, 2008). In this thesis and the research of Kiers *et al.* the same beneficial effects for fermented soya beans were found, in which hydrolysis takes place by the enzymes of the moulds (Kiers *et al.*, 2006; Kiers *et al.*, 2007).

Anti-adhesion bioactivity in a broader perspective
In this thesis most of the work was performed on piglet brush border cells with a target ETEC strain as a model to investigate adhesion inhibition. This strain was selected, because it was used previously in the *in vivo* piglet study (Kiers *et al.*, 2003). The question arose whether the observed adhesion inhibition observed could also be detected with other diarrhoea causing strains in piglets. Moreover, could tempe also be used to prevent diarrhoea in humans?
Chapter 4 dealt with the adhesion of different ETEC strains isolated from piglets with diarrhoea to brush border cells, and the inhibition of adhesion by tempe extracts. Tempe extracts decreased the adhesion of most ETEC tested on piglet brush border cells. Consequently tempe extracts could prevent intestinal cells being colonized by different strains of ETEC causing diarrhoea in piglets.
Furthermore, several strains with different serotypes of *E. coli* were collected and tested for their adhesion inhibition to piglet brush border cells and Caco-2 human intestinal epithelial cells. Especially the different human ETEC strains with known colonization factors (CS) were of interest, because of the possible interaction of the

tempe extracts with these specific colonization factors, since ETEC is an important causative organism in childhood diarrhoea (Bhan, 2000; Qadri *et al.*, 2005).

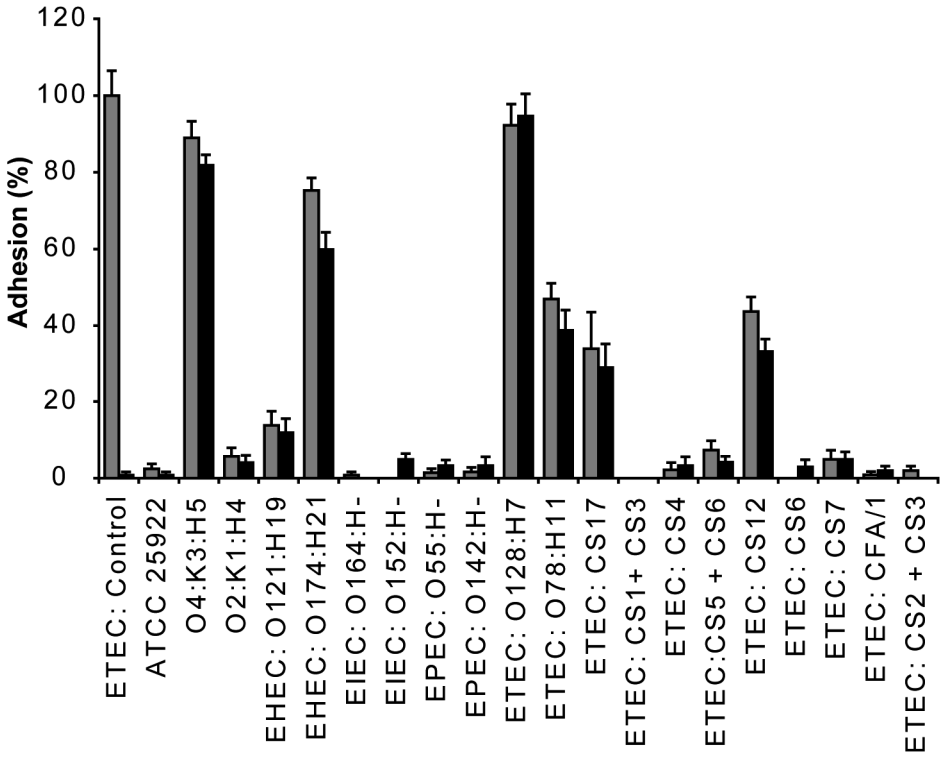


Figure 2: Adhesion of different human *E. coli* strains to piglet intestinal brush border cells (gray bars) and the adhesion of these strains with addition of tempe extracts (black bars). The strains were obtained from the following places: ETEC: control: piglet K88-positive O149:K91 (ID 1000), ID-Lelystad, Lelystad, The Netherlands; ATCC25922, O4:K3:H5 and O2:K1:H4 from VWA, Zutphen, The Netherlands; EHEC: O121:H19 (1120700042), EHEC: O174:H21 (1120700050), EIEC: O164:H- (ECOL396), EIEC: O152:H- (ECOL384), EPEC: O55:H- (ECOL280), EPEC O142:H- (ECOL372), ETEC: O128:H7 (ECOL522) and ETEC: O78:H11 (ECOL402) from RIVM, Utrecht, The Netherlands; ETEC CS17 (E20738A), ETEC CS1+CS3 (E1392-75), ETEC CS4 (E11881/9), ETEC CS5 + CS6 (VM75688), ETEC CS12 (350C1A), ETEC CS6 (E11881/14), ETEC CS7 (E29101A), ETEC CFA/I (258909-3), ETEC CS2 + CS3 (278485-2) from University of Gothenburg, Gothenburg, Sweden. Bars represent mean values of 12 measurements, expressed as % adhesion compared to the control without tempe addition. Error bars represent SEM.

Figure 2 shows the adhesion of these *E. coli* to piglet brush border cells and the adhesion with addition of tempe extracts. Three strains showed adhesion to the brush border cells in the same order as the positive control. This positive control was used in earlier brush border experiments as reference strains. Whereas three strains showed some adhesion to the brush borders cells, the other fourteen strains did not show any adhesion to the piglet brush border cells. After addition of tempe extracts, none of the strains showed inhibition of adhesion. This suggests that the tempe extract was not bioactive against the colonization of these strains in the intestinal cells. The strains tested were of human origin from different categories of diarrhoeagenic bacteria, but for all of them adhesion is an important step in pathogenisis. Since the brush borders are from piglets and not from humans, it is possible that the specific adhesion-ligand interaction can not be formed. This is indeed indicated by the low number of *E. coli* strains that are capable to adhere to piglet brush border cells.

Subsequently, some diarrhoea causing *E. coli* strains were selected and tested for their adhesion inhibition to the human Caco-2 intestinal epithelial cells. All tested strains showed adhesion to the Caco-2 cells (data not shown) and the inhibition of this adhesion is shown in figure 3.

The piglet positive control, strain ATCC 29255 and EIEC: O164:H- showed an inhibition of adhesion up to 70%. The different human ETEC strains with known colonization factors (CS) did not show any inhibition of adhesion by the tempe extracts. One of them (ETEC: CS6) even showed a higher adhesion value after addition of the tempe extract.

Nevertheless, we found three (two and the control) *E. coli* strains of which the adhesion to intestinal Caco-2 cells was inhibited by tempe extract. However, presently no further conclusions about the specific interactions between *E. coli* and tempe within humans can be drawn.

The anti-diarrhoeal effect of tempe was demonstrated in several human studies with children suffering from diarrhoea (Mahmud *et al.*, 1985; Kalavi *et al.*, 1996; Karyadi and Lukito, 1996; Soenarto *et al.*, 1997). Since the organisms causing diarrhoea in these studies were not known, a specific study to explain the effect of tempe on these bacteria was not possible. The nutritional status also has an potential impact on diarrhoeal episodes. The interactions between diarrhoea and malnutrition as a cause or an effect are well recognised (Gracey, 1996; Gadewar and Fasano, 2005). Tempe might have potential in breaking this vicious cycle of malnutrition and diarrhoea, since it is nutritious, easily digestible and absorbable and might also protect against some types of diarrhoea in humans.

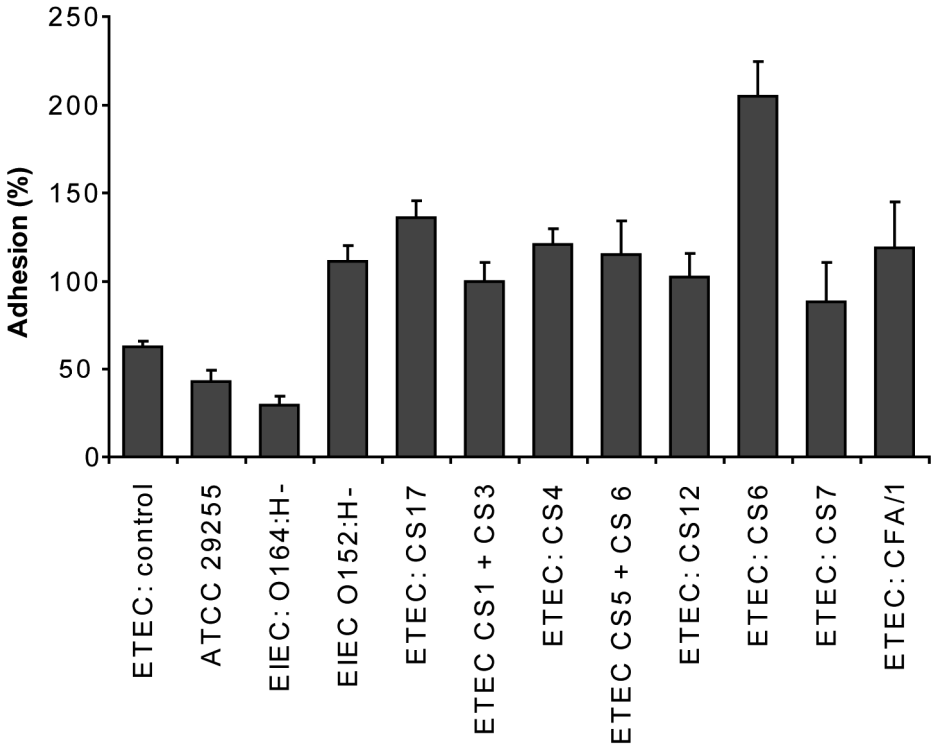


Figure 3: Adhesion of different *E. coli* strains to Caco-2 intestinal epithelial cells. Bars represent mean values, expressed as % adhesion compared to the adhesion of the strain without tempe addition. Error bars represent SEM. The strains were collected from the following places, in brackets the number of replicates is mentioned: ETEC: control: (n=22) piglet K88-positive O149:K91 (ID 1000) from ID-Lelystad, Lelystad, The Netherlands; ATCC25922 (n=6), from VWA, Zutphen, The Netherlands; EIEC: O164:H- (n=6) (ECOL396), EIEC: O152:H- (n=3) (ECOL384) from RIVM, Utrecht, The Netherlands; ETEC CS17 (n=3) (E20738A), ETEC CS1 + CS3 (n=6) (E1392-75), ETEC CS4 (n=3) (E11881/9), ETEC CS5 + CS6 (n=3) (VM75688), ETEC CS12 (n=6) (350C1A), ETEC CS6 (n=3) (E11881/14), ETEC CS7 (n=2) (E29101A) and ETEC CFA/I (n=3) (258909-3) from University of Gothenburg, Gothenburg, Sweden.

Conclusions and future perspectives

During this research tempe was found to be bioactive in two ways towards diarrhoea-associated bacteria. On the one hand, tempe inhibits the adhesion of ETEC to intestinal cells, which can be of interest in the recovery and prevention of diarrhoea in piglets as

well as in humans. On the other hand, tempe is antibacterial against *B. cereus* cells and spores, which can be of interest in food preservation and pathogen control.

The anti-adhesion activity is caused by an interaction between ETEC and tempe extracts, which results in a loss of adhesion capability of ETEC to the intestinal cells. This bioactivity is found in tempe derived from leguminous seeds, whereas tempe derived from cereals is inactive. The bioactive component(s) is / are released or formed during fermentation by enzymatic degradation of leguminous matter. Fermentation with several other microorganisms also resulted in the formation of bioactive component(s).

Furthermore, the bioactive component is of carbohydrate nature, and contains arabinose as an important monosaccharide constituent. The bioactive component is supposed to originate from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of legumes.

Further characterization of the anti-adhesion component is needed to fully understand the mechanism of action. A well purified and characterized bioactive component needs to be tested in animal and human studies to check if the *in vitro* results can be translated to the *in vivo* situation. Further research is required to exploit potential application of the bioactive principle in food or feed matrices via the addition of specific enzymes liberating the arabinose containing medium-weight polysaccharides.

In addition, a characterization of the antibacterial component in tempe by chemical analyses will be required to assess its potential in the food industry. A full characterization could open new possibilities for producing the bioactive component and application in food preservation and pathogen control.

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Summary

For many thousands of years, food has been fermented. Examples of fermented food are: beer, bread, wine, sausages and yoghurt. Fermentation means the modification of biological material using microorganisms with the purpose to get a desirable product. Fermentation is used for preservation, improvement of the flavour and texture and nutritional enrichment of the products.

Tempe is a collective name for legumes, cereals and other biological materials, fermented by a mould. This technique has its origin in Indonesia. Soya beans are the most commonly used substrate for the tempe production. During fermentation, the soya beans are bound together by the mycelium of the mould into a compact cake and macronutrients are enzymatically degraded.

Previous research showed that the duration and severity of diarrhoea was less in piglets and children when tempe was consumed. In this thesis, this bioactivity of tempe is further described. The mode of action and the chemical characteristics of the bioactive component were studied. Furthermore, the specificity of the bioactive component was investigated. In addition, the effect of the use of various substrates and/or starter cultures was tested.

In this research, a method to investigate the effect of tempe on diarrhoea at laboratory scale was developed. This method was developed without the use of laboratory animals could be used, the tested methods are descried in chapter 2. Firstly, the effect of tempe extracts on the growth of enterotoxigenic *E. coli* was investigated. However, the growth of these bacteria was not influenced by the addition of tempe extract. Another method to measure the influence of tempe on diarrhoea is the prevention of adhesion of the ETEC to intestinal cells. Two *in vitro* models, which measure the adhesion of bacteria to intestinal cells were tested: (a) brush border cells of piglet intestines and (b) the human intestinal cell culture Caco-2. The adhesion of bacteria to intestinal cells is an important step in the infection of humans and animals. If bacteria cannot adhere to the epithelial cells, they cannot colonize and cannot cause infection. The adhesion of ETEC to the brush border cells was strongly inhibited by the addition of tempe extracts. Addition of cooked soya beans resulted in a partial inhibition (50%) to the brush border cells. On the other hand, the addition of cooked soya bean to the Caco-2 intestinal epithelial cells did not inhibit the adhesion. However, the adhesion of ETEC to Caco-2 cells was inhibited by about 50% after addition of the tempe extract.

Furthermore, chapter 2 described in which way the adhesion can be inhibited by the tempe extracts. Pre-incubation of ETEC with tempe extracts, followed by washing off

the unbound tempe extracts, resulted in a full inhibition of adhesion to the brush border cells. On the other hand, pre-incubation of brush border cells with tempe, followed by washing off the unbound tempe extract, resulted in adhesion of the ETEC to the brush border cells. Tempe appeared to interact with the ETEC and as a result the ETEC could not bind to the intestinal cells.

Subsequently, the bioactivity of tempe against other diarrhoea-associated microorganism was investigated. Tempe could inhibit the growth of various Gram-positive bacteria. This antibacterial effect was further investigated with *Bacillus cereus* and described in chapter 3. After exposure of *B. cereus* to tempe extracts a rapid reduction of 4 Log CFU ml^{-1} vegetative cells was observed within 15 minutes. The antibacterial activity of tempe was also measured with optical density measurements, which measured no growth of *B. cereus* during 8 hours (or longer). The tempe extracts could also inactivate the *B. cereus* spores. This happened just upon germination of the spores, in a way that they could not grow out. Fluorescence and phase contrast microscopy showed that the inactivation of the spores was caused by the permeabilisation of the cytoplasmic membrane. Cooked soya beans and mould grown on various media did not show antibacterial activity. The antibacterial activity is released during the fermentation of the soya beans, presumably by the enzymatic degradation of the macronutrients. The component responsible for the antibacterial activity was sensitive for heating and for protease treatments. Also after lowering the pH of the tempe extract till pH 4 or lower the antibacterial effect was lost. This showed that the antibacterial effect is caused by an proteinaceous component from tempe.

The next chapters of this thesis dealed with the anti-adhesion effect, which was found in chapter 2. In chapter 4 the effect of tempe on various ETEC isolated from infected piglets was tested. The tempe extracts reduced the adhesion of different ETEC strains to the brush border cells completely. Furthermore, this chapter described the use of various substrates and starter cultures for the fermentation and their effects on the anti-adhesion activity. All fermented legumes could inhibit the adhesion, whereas the fermented cereals could not inhibit the adhesion. During incubation of soya beans with various moulds, *Bacillus* spp. and some yeasts, the macronutrients were degraded. These products could also prevent the adhesion of ETEC to brush border cells. The incubation with *Lactobacillus* and some yeasts showed no or only a little degradation of macronutrients of the soya beans. These products also showed no bioactivity. This showed that the anti-adhesion activity of tempe is released during the fermentation by enzymatic degradation of macronutrients of legumes by a variety of microorgansims.

In chapter 5, the chemical composition of the bioactive anti-adhesion component in tempe was studied in more detail. After defatting, heating and protease treatment the tempe extracts remained bioactive, whereas the incubation of tempe with polysaccharide degrading enzymes nullified the bioactivity. The bioactive component is larger than 30 kDa. This all suggests that carbohydrate structures are responsible for the bioactivity of tempe.

The tempe extracts, which bioactivity was reduced after incubation with polysaccharide degrading enzymes, were dialyzed to remove degraded sugars. From the remaining material the monosaccharide composition was determined. This showed that the reduction in bioactivity was caused by an reduction of the monosaccharide arabinose. Further purification of the active component resulted in an active fraction with an increased concentration of carbohydrates to 56%, of which half of it consists of arabinose. Arabinose is mainly present in the arabinan or arabinogalactan side chains of the pectic cell wall polysaccharides of the soya beans. A partial degradation of these cell wall polysaccharides is important to obtain the tempe bioactivity.

The interaction shown between ETEC and tempe, which is described in chapter 2 is in agreement with these findings, because such an interaction was shown to exist between a carbohydrate and the fimbriae of the *E. coli*.

In conclusion, in this thesis the tempe extracts showed two different bioactive effects, namely: (a) the antibacterial activity against *B. cereus* cells and germinating spores and (b) the anti-adhesion activity of ETEC to intestinal cells. The antibacterial activity is caused by a protein component, whereas the anti-adhesion activity is caused by a carbohydrate component. Both bioactive activities are formed or released during the fermentation. The degradation of macronutrients by the enzymes of the microorganisms is of great importance. Further characterization of the bioactive components is needed for a successful introduction in products. The possibilities for (synthetic) production of the bioactive components can be considered.

Samenvatting

Al vele duizenden jaren worden levensmiddelen gefermenteerd. Voorbeelden van gefermenteerde levensmiddelen zijn: bier, brood, wijn, worsten en yoghurt. Fermentatie betekent het omzetten van biologische materialen met behulp van micro-organismen met het doel een aantrekkelijk product te verkrijgen. Fermentatie wordt gebruikt ter verlenging van de houdbaarheid, verbetering van de geur, smaak en textuur en verhoging van de voedingswaarde van levensmiddelen.
Tempe is een verzamelnaam voor peulvruchten, granen of andere biologische materialen, die gefermenteerd worden met een schimmel. Deze techniek is oorspronkelijk afkomstig uit Indonesië. Sojabonen zijn het meest gebruikte substraat voor het produceren van tempe. Tijdens fermentatie bindt het mycelium van de schimmel de sojabonen samen tot een compacte cake en vindt enzymatische afbraak van de macronutriënten van de sojabonen plaats.
Voorgaand onderzoek heeft aangetoond dat de consumptie van tempe de duur en de ernst van diarree kan remmen bij biggen en kinderen. In dit proefschrift is deze bioactiviteit van tempe verder beschreven. Het werkingsmechanisme en de chemische eigenschapen van de bioactieve stof zijn nader onderzocht. Verder is ook gekeken of de bioactiviteit specifiek is voor gefermenteerde sojabonen, of dat fermentatie breder toegepast kan worden, door het gebruik van andere substraten en/of starter cultures.
In dit onderzoek is gezocht naar een methode, waarmee de remming van diarree door tempe kan worden gemeten op laboratoriumschaal. Hierbij wordt geen gebruik gemaakt van proefdieren; de gebruikte methoden zijn beschreven in hoofdstuk 2. In eerste instantie is gekeken of de groei van Enterotoxigene *Escherichia coli* (ETEC) bacteriën geremd kan worden in aanwezigheid van tempe extracten. Echter, de groei van de bacteriën bleek even snel te zijn als de groei zonder toevoeging van tempe-extracten. Een andere manier waarop tempe diarree zou kunnen remmen is door het voorkómen van adhesie (binding) van de bacteriën aan darmcellen. Twee *in vitro* modellen, die de adhesie van bacteriën aan darmcellen meten, werden getest: (a) brushbordercellen van biggendarmen en (b) de humane darmepitheelcellijn Caco-2. De adhesie van bacteriën aan darmcellen is een belangrijke stap in de infectie bij mens en dier. Wanneer de adhesie wordt voorkómen kunnen de bacteriën niet koloniseren, waardoor infectie en dus het ziekworden uitblijft. De adhesie van ETEC aan de brushbordercellen werd volledig geremd door de toevoeging van tempe-extracten. Toevoeging van een extract met gekookte sojabonen leverde een gedeeltelijke remming (50%) van de adhesie aan de brushbordercellen op. Daarentegen leverde toevoeging

van gekookte sojabonen aan humane Caco-2 darmepitheelcellen geen afname in de adhesie op. De adhesie van ETEC aan de Caco-2 cellen nam wél met ongeveer 50% af na toevoeging van tempe extract.
Verder is in hoofdstuk 2 beschreven op welke wijze de adhesie geremd kan worden door de tempe-extracten. Vóór-incubatie van ETEC met tempe extracten, waarna het ongebonden tempe-extract werd weggewassen, resulteerde in een volledige afname van de adhesie aan de brushbordercellen. Daarentegen resulteerde voor-incubatie van brushbordercellen met tempe, waarna het ongebonden tempe extract werd weggewassen, wel tot adhesie van de ETEC aan de brushbordercellen. Hieruit is gebleken dat de tempe extracten een interactie aangaan met de ETEC, waardoor deze niet meer in staat zijn om zich aan de darmcellen te binden.
Vervolgens is gekeken of tempe ook bioactief is tegen andere bacteriën, die diarree zouden kunnen veroorzaken. Tempe bleek de groei van verschillende Gram-positieve bacteriën te kunnen remmen. Dit antibacteriële effect is verder onderzocht op *Bacillus cereus*-cellen en -sporen in hoofdstuk 3. Na blootstelling van *B. cereus* aan tempe-extracten vond binnen 15 minuten een afname in levende *B. cereus* cellen plaats van 4 log KVE ml^{-1}. De antibacteriële activiteit van tempe werd ook gemeten met optische-dichtheid experimenten, waarbij er gedurende 8 uur (of langer) geen groei van *B. cereus* cellen optrad. Ook bleken de tempe-extracten in staat om *B. cereus* sporen te inactiveren, net na hun ontkieming, zodat deze niet meer in staat waren om uit te groeien. Fluorescentie- en fasecontrastmicroscopie wezen uit dat de inactivatie van de sporen veroorzaakt wordt door permeabilisatie van het cytoplasmatisch membraan. Gekookte sojabonen en schimmel, gegroeid op verschillende media, vertoonden geen antibacteriële activiteit. De antibacteriële activiteit is ontstaan gedurende de fermentatie van de sojabonen, waarschijnlijk door enzymatische afbraak van de macronutriënten. De component die verantwoordelijk is voor de antibacteriële werking bleek gevoelig te zijn voor verhitting en proteasebehandeling. Ook na verlaging van de pH van het tempe-extract naar pH 4 of lager ging de antibacteriële activiteit verloren. Dit wijst erop, dat het antibacteriële effect wordt veroorzaakt door een eiwit-bevattende component in tempe.
De volgende hoofdstukken van dit proefschrift gaan verder in op het anti-adhesie effect dat werd gevonden in hoofdstuk 2. In hoofdstuk 4 is het effect van tempe op verschillende ETEC isolaten uit geïnfecteerde biggen getest. De tempe-extracten bleken de adhesie van verschillende ETEC isolaten aan brushbordercellen te kunnen voorkómen. Verder zijn in dit hoofdstuk het gebruik van verschillende substraten en startercultures voor de fermentatie en de effecten hiervan op de anti-adhesie activiteit

getest. Alle gefermenteerde peulvruchten bleken de adhesie te kunnen remmen, terwijl de gefermenteerde granen hier niet toe in staat bleken. Tijdens de incubatie van de sojabonen met de verschillende schimmels, *Bacillus* spp. en enkele gisten vond afbraak plaats van de macronutriënten van de sojabonen. Deze producten bleken ook in staat te zijn om de adhesie van ETEC aan brushbordercellen te voorkómen. De incubatie met *Lactobacillus* en enkele gisten vertoonde geen of slechts een geringe afbraak van de macronutriënten van de sojabonen. Deze producten vertoonden ook geen bioactiviteit. Hieruit blijkt dat de anti-adhesie activiteit van tempe wordt gevormd tijdens de fermentatie door de enzymatisch afbraak van macronutriënten uit peulvruchten.

In hoofdstuk 5 is dieper ingegaan op de chemische samenstelling van de bioactieve anti-adhesie component in tempe. Na ontvetting, verhitting en proteasebehandeling bleven de tempe-extracten bioactief, terwijl de incubatie met enkele polysaccharide-splitsende enzymen er toe leidde dat de bioactiviteit verloren ging. Ook bleek de bioactieve component groter dan 30 kDA te zijn. Dit duidt erop dat koolhydraatstructuren verantwoordelijk zijn voor de bioactiviteit van tempe. De afgebroken suikers werden verwijderd met behulp van dialyse en vervolgens werd van het overgebleven materiaal de monosaccharide-samenstelling bepaald. Hieruit werd duidelijk dat de afname van de bioactiviteit werd veroorzaakt door een afname van de monosaccharide arabinose. Verdere zuivering van de actieve component resulteerde in een actieve fractie met een toegenomen concentratie aan koolhydraten tot 56%, waarvan de helft uit arabinose bestaat. In tempe is arabinose voornamelijk aanwezig in de arabinaan- of arabinogalactaan-zijketens van de pectinecelwandpolysacchariden van de sojabonen. Een gedeeltelijke afbraak van deze celwandpolysacchariden is belangrijk om bioactieve tempe te verkrijgen. De aangetoonde interactie tussen ETEC en tempe, die beschreven werd in hoofdstuk 2 komt overeen met deze bevindingen, omdat zo'n interactie vaak een binding is van een koolhydraat aan de fimbriae van de *E. coli*.

Concluderend, tempe extracten laten in dit proefschrift twee verschillende bioactieve effecten zien, te weten: (a) de antibacteriële activiteit tegen *B. cereus* cellen en ontkiemde sporen; en (b) de anti-adhesie activiteit van ETEC aan darmcellen. De antibacteriële activiteit wordt veroorzaakt door een eiwitcomponent, terwijl de anti-adhesie wordt veroorzaakt door een koolhydraatcomponent. Beide bioactieve activiteiten ontstaan of komen vrij gedurende de fermentatie. De afbraak van de macronutriënten, die veroorzaakt wordt door de enzymen van de microorganismen is hierbij van belang. Verdere karakterisering van de bioactieve componenten is nodig, voor een succesvolle toepassing ervan in producten. Ook kan dan bekeken worden of een (synthetische) productie van de bioactieve componenten uit tempe mogelijk is.

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List of Publications

Becker, P.M., Galetti, S., Roubos-van den Hil, P.J., Van Wikselaar P.G. (2007) Validation of growth as measurand for bacterial adhesion to food and feed ingredients, *J Appl Microbiol* **103**, 2686-2696.

Dommels, Y.E.M., Van den Hil, P.J., Linssen, J.P.H., Alink, G.M., Van Bladeren, P.J., Van Ommen, B. (2003) High fat fish oil diets modulate expression of genes involved in oxidative stress, cell growth, acute phase response and lipid metabolism in normal colonic eptihelium male F344 rats, In *Effects of n-6 and n-3 polyunsaturated fatty acids on colorectal carcinogenesis*. Dommels, Y.E.M. pp. 105-133: PhD thesis, Wageningen University, Wageningen, The Netherlands.

Hornstra, L.M., Van der Voort, M. Wijnands, L.M., Roubos-van den Hil, P.J., Abee, T. (2009) Role of germination receptors in Caco-2 cell-initiated germination of *Bacillus cereus* ATCC 14579 endospores, *Appl Environ Microbiol* **75,** 1201-1203.

Roubos-van den Hil, P.J., Nout, M.J.R., Beumer, R.R., Van der Meulen J., Zwietering, M.H. (2009) Fermented soya bean (tempe) extracts reduce adhesion of enterotoxigenic *Escherichia coli* to intestinal epithelial cells, *J Appl Microbiol* **106,** 1013-1021.

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Roubos-van den Hil, P.J., Schols, H.A., Nout, M.J.R., Zwietering, M.H., Gruppen, H., (2010) A first characterization of bioactive components in soya bean tempe that protect human and animal intestinal cells against ETEC infection. *J Agric Food Chem **58**, 7649-7656.*

Curriculum Vitae

Petra Johanna Roubos was born on November 13th 1979 in Brakel, The Netherlands. She finished her secondary education, receiving her VWO in 1998 at "De Lage Waard" in Papendrecht. From 1998 until 2004, she studied both Food Science and Human Nutrition at Wageningen University. As part of her MSc studies research projects were carried out within the Food Chemistry and Toxicology and Public Health and Nutrition specializations. Furthermore, two internships were carried out: One industrial at Nutricia, Zoetermeer, The Netherlands and a research internship at RIKILT – Institute of Food Safety, Wageningen, The Netherlands. In January 2005, she started her PhD project entitled: "Bioactive components of fermented soya beans effective against diarrhoea-associated bacteria" at the Laboratory of Food Microbiology and the Laboratory of Food Chemistry of Wageningen University. The results of this project are described in this thesis. From August 2010, Petra is employed as researcher at the Ingredients Research Centre of Nutreco, Boxmeer, The Netherlands.

Overview of completed training activities

Discipline specific activities

Courses

Socrates Intensive Programme Food & Health, VLAG, Paris, 2005
Glycosciences, VLAG, Wageningen, 2006
Industrial Proteins, VLAG, Wageningen 2006
Ecophysiology of the gastro-intestinal tract, VLAG, Wageningen, 2007
Genetics and physiology of food-associated microorganism, VLAG, Wageningen, 2007
Food Fermentation, VLAG, Wageningen, 2008

Meetings

Alternatieven voor voederantibiotica; zoötechniek en voedselveiligheid, ILVO, Gent, 2006 International conference Soy & Health, Düsseldorf, 2006
NVVM scientific spring meeting, Arnhem, 2007 and 2009
9th Annual Gut Day, Gut Flora Foundation, Wageningen, 2007
Food Microbiology Congress, Aberdeen, Ireland, 2008

General courses

VLAG PhD week, Bilthoven, 2005
Techniques for Writing and Presenting a Scientific Paper, WGS, Wageningen, 2008

Other activities

Preparation of the PhD research proposal, 2005
General meetings Laboratory of Food Microbiology, 2005-2010
PhD-trip, Laboratory of Food Microbiology, South Africa, 2005
Protein meetings, Laboratory of Food Chemistry, 2006-2008
Supervising BSc an MSc students, 2006-2009
Organization of PhD-trip to Canada, 2008
PhD-trip, Laboratory of Food Microbiology, Canada, 2008

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