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Anti-Diarrhoeal Aspects of Fermented Soya Beans

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1. Introduction

Generally, fermentation can be described as the modification of biological material using microorganisms with the purpose to obtain a desirable product. 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 of food, (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).
During the fermentation processes, enzymes that are synthesized by the microorganisms are important, since they carry out reactions, which contribute to the development of specific textures, tastes and aromas.
Many fermented products especially fermented soya bean products, find their origin in Asia. Fermented soya bean foods are attractive products and enjoy an increasing interest because of their nutritional and health benefits. One of these products is tempe. 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 in which the macronutrients are enzymatically degraded by the fungal enzymes.
In this chapter, firstly a description will be given of the product tempe. Processing, composition and fermentation characteristics will be described. Also different health effects of the product will be discussed with a special focus on the anti-diarrhoeal aspects of tempe. The second part of the chapter will focus on the anti-diarrhoeal aspects in more detail. The mode of action and the chemical characteristics of the bioactive component will be

discussed. Finally we will discuss the possibilities for application of the product in food or feed industry.

2. Tempe

2.1 Definition

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 & Kiers, 2005).

Fig. 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 & 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 (Astuti, 2000; Nout & Kiers, 2005; Steinkraus, 1996).
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.

2.2 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 & 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 & 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 & Hesseltine, 1979; Nout & Kiers, 2005; Nout & Rombouts, 1990; Shurtleff & Aoyagi, 2001; Steinkraus, 1996; Tibbott, 2004). 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 & 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 & 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.

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

2.3 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 & Busse, 1991), cowpea (Egounlety, 2001; Kiers et al., 2000), groundbean (Egounlety, 2001), horse bean (Ashenafi & Busse, 1991), lima bean (Ko & Hesseltine, 1979), pea (Ashenafi & Busse, 1991), oats (Eklund-Jonsson *et al.*, 2006), sorghum (Mugula & Lyimo, 2000) and wheat (Hachmeister & Fung, 1993) have been reported to be suitable substrates. Some substrates can only be processed in combination 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 (Feng, 2006; Nout & Kiers, 2005).

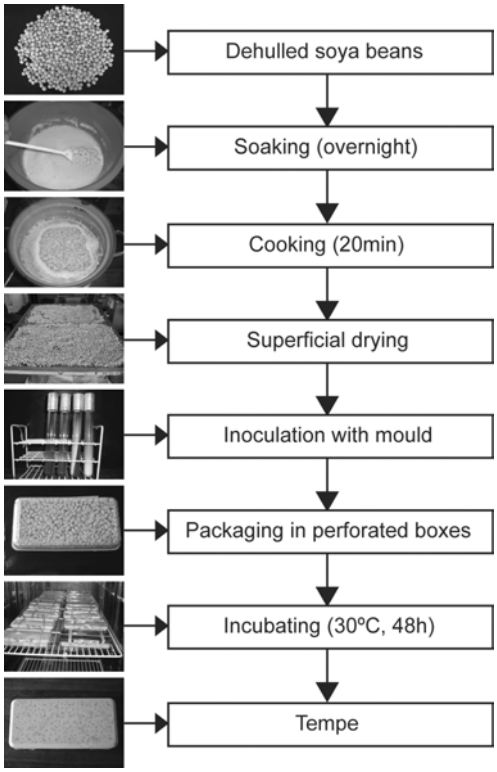

Fig. 3. Production process of soya bean tempe.

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.

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} of cooked beans. Traditionally, the beans are packed in punctured banana leaves allowing a limited supply of air to the beans (figure 4).

Fig. 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 25-30°C. 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 (Nout & Kiers, 2005; Nout & Rombouts, 1990; Shurtleff & Aoyagi, 2001; Steinkraus, 1985; Steinkraus, 1996).

2.4 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 & Kiers, 2005).

Lactic acid bacteria play a role in the acidification of the soya beans during soaking, thereby preventing the growth of spoilage microorganisms (Ashenafi & Busse, 1991; Nout *et al.*, 1987) 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 & Rombouts, 1990).

2.5 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 substances of lower molecular mass, with a higher water-solubility. Also vitamins, phytochemicals and anti-oxidative constituents are formed (Astuti, 2000; Nout & 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.

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

Table 1. Composition of cooked soya beans and tempe.

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 phenomenon can be related to its chemical structure (De Reu et al., 1995; Nowak, 1992). The degree of hydrolysis strongly depends on the fungal strain and the fermentation conditions.

The concentration 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 & Owens, 1996).

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 (Egounlety & Aworh, 2003; Mulyowidarso *et al.*, 1991). 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., 2000). 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 (Astuti, 2000; Egounlety & Aworh, 2003; Nout & Kiers, 2005; Prinyawiwatkul *et al.*, 1996; Tawali *et al.*, 1998). 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; Denter *et al.*, 1998; Keuth & Bisping, 1993).

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 (Murphy *et al.*, 1999; Wang & Murphy, 1996). 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).

2.6 Health aspects of tempe

The effects of soya beans on the health of man and animals have 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 (Balk et al., 2005; Messina et al., 2002). 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., 2000). 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 (Astuti, 2000; Karyadi & Lukito, 1996; Karyadi & 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.

3. Tempe and diarhoea

3.1 Antibacterial effects of tempe

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. (Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969). These studies suggest the presence of a component from the tempe or the *Rhizopus* spp, that inhibits the growth of these bacteria.
Recently we investigated the antibacterial activity of tempe extracts towards a range of bacterial strains 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. 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 (Kiers et al., 2002; Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969).

Strain	Growth delay (h)
Listeria innocua	1.6
Listeria monocytogenes	1.0
Lactobacillus bulgaricus	>22[2]
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] Tempe extract (1% w/v) was prepared by solubilizing 10 g l^{-1} dry soluble material in BHI.
[2] > Means that the growth delay is longer than the total measurement period.

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

Further research on the antibacterial effect towards *Bacillus* strains was described in Roubos–van den Hil et al. (2010a). In this article it is shown that 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.

3.2 *In vivo* effects of tempe on diarrhoea

The role of tempe, as part of the diet and the effect on the development of diarrhoeal infection in animals (table 3a) and humans (table 3b) was investigated by several researchers. 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 3.
Table 3a 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 3b 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.
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.
The fermented soya beans are especially of interest in patients suffering from intestinal digestive defects. The combination of the high digestibility and nutritional value makes the food important 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 & 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 & Aworh, 2002).

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 3a. Effects of tempe on *E. coli* infection in animals.

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
Partawiharja, (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

Table 3b. Effects of tempe on human diarrhoea patients.

3.3 The influence of tempe on the adhesion of ETEC to intestinal cells

Enterotoxigenic *Escherichia coli* 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 & 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 (Bhan, 2000; Dalton et al., 1999). Many similarities can be found in the pathogenisis by ETEC infections of animals and humans; this provides opportunities to understand human ETEC infections by the use of animal models (Nagy & Fekete, 2005).

Some of the *in vivo* studies described in paragraph 3.2 showed a specific effect on *E. coli* induced diarrhoea, but in paragraph 3.1 we discussed the antibacterial effect of tempe not to be antibacterial towards *E. coli* bacteria. The anti-diarrhoeal effect is probably not a result of a growth inhibiting effect of the bacteria. Another 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 (Nagy & Fekete, 2005; Nataro & Kaper, 1998).

Indeed, i*n vitro* research showed a strong inhibition of the adhesion of Enterotoxigenic *Escherichia coli* (ETEC) to piglet intestinal cells after addition of tempe extracts (Kiers *et al.*, 2002). Further research indicated 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 (Roubos-van den Hil et al., 2009).

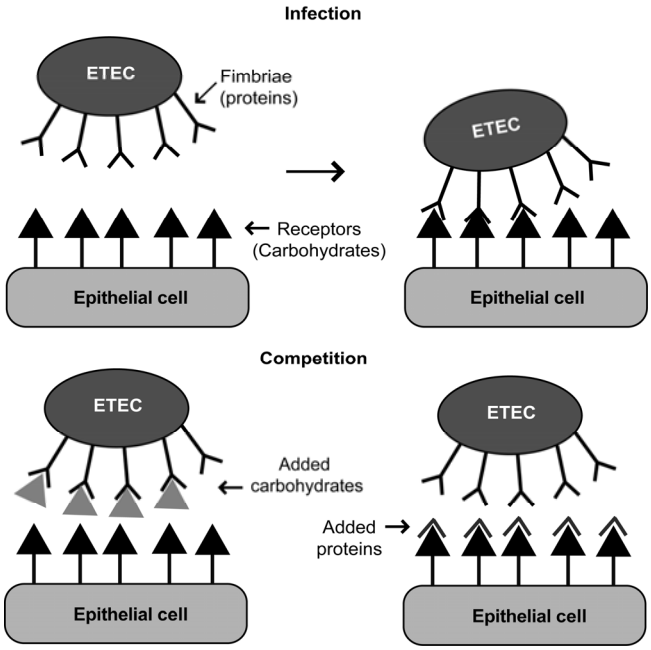

Fig. 5. Infection of intestinal tissue cells by ETEC bacteria and the mechanisms of inhibition of adhesion by specific food components.

The question remains how a fermented soya extract can prevent intestinal cells for adhesion. It is known that 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 5. Carbohydrates, which are structurally similar to the binding sites of the epithelial cells, can adhere to the bacteria (Ofek et al., 2003; Pieters, 2007; Sharon, 2006). 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).

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 proteinaceous fimbriae. For example, bromelain, a proteolytic enzyme from pineapple stems, reduced ETEC adhesion to intestinal cells significantly (Chandler & Mynott, 1998). However, Roubos-van den Hil et al. (2010c) showed that after heating, during which the proteolytic enzymes were inactivated, the tempe extracts remain bioactive. Thus, proteolytic enzymes could not be responsible for the adhesion inhibition of tempe extracts.

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.

Table 4. Adhesion of ETEC to brush border cells, as affected by composition and timing of reaction mixture (Roubos-van den Hil et al., 2009).

Further research about the interactions between intestinal epithelial cells, ETEC and tempe extracts was performed. Pre-treatment of ETEC with tempe extracts resulted in strongly reduced adhesion (table 4). 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 (Roubos-van den Hil et al., 2009).

3.4 Chemical characteristics of the anti-adhesion component in tempe

The anti-adhesion activity of fermented soya beans is expected to be mediated by a specific component or specific structure. Roubos-van den Hil et al. (2010b) investigated the effect of various fermentation substrates on the inhibition of ETEC adhesion to intestinal epithelial cells. 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. It was concluded that 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. 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 (Nout & Rombouts, 1990; Samson *et al.*, 1987), of which less is known in relation to their functions in tempe. Research was done on the fermentation of soya beans with several pure cultures of microorganisms, isolated from tempe or other fermented products, to detect bioactivity. The *Bacillus* spp., moulds and some yeast 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 (Roubos-van den Hil et al., 2010b).
Further chemical characterization experiments were performed by Roubos van den Hil et al. (2010c). 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, 2007), 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 found by Roubos-van den Hil et al., (2009), indicating that the inhibition of adhesion is caused by an interaction between ETEC and tempe extracts, suggest that the bioactive component in tempe is of carbohydrate nature.

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 the bioactivity of the fermented extracts was measured in water-soluble extracts (Roubos-van den Hil et al., 2009), an 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 (Roubos van den Hil et al., 2010c), which corresponds to the observation of the predominant degradation of pectin and arabinogalactan during fermentation of soya beans (De Reu *et al.*, 1997).

Roubos-van den Hil et al (2010c) treated the bioactive tempe extract 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.

	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).

Table 5. Monosaccharide composition (mol %) of leguminous and cereal grains fermented with *Rhizopus microsporus* (LU 573) after hydrolysis.

The monosaccharide composition of leguminous and cereal grains was determined (table 5). 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 support our conclusion that the bioactive component has to contain at least arabinose.

Arabinose is an important constituent of pectic cell wall polysaccharides of soya beans. It is especially present in the rather long arabinan and (arabino)galactan side chains of rhamno-galacturonans (Huisman, 2000). We hypothesize that some structural epitopes of such arabinans or arabinogalactans are responsible for the bioactivity in tempe. During fermentation these structures become more soluble and thus 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 & 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 (Kiarie et al., 2007; Meng et al., 2005; Pluske et al., 2002). 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). These results can be compared with the results in this chapter that discuss the effect of hydrolysis that takes place by the enzymes of the moulds.

3.5 Anti-adhesion bioactivity in a broader perspective

Most of the results described in this chapter were performed with *in vitro* studies with a specific target ETEC strain as a model to investigate adhesion inhibition. 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?
Roubos- van den Hil et al. (2010b) described 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 also an important causative organism in childhood diarrhoea (Bhan, 2000; Qadri *et al.*, 2005).
Figure 6 shows the adhesion of these *E. coli* strains to piglet brush border cells and their adhesion in the presence 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 a reference strain. 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 piglet 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.

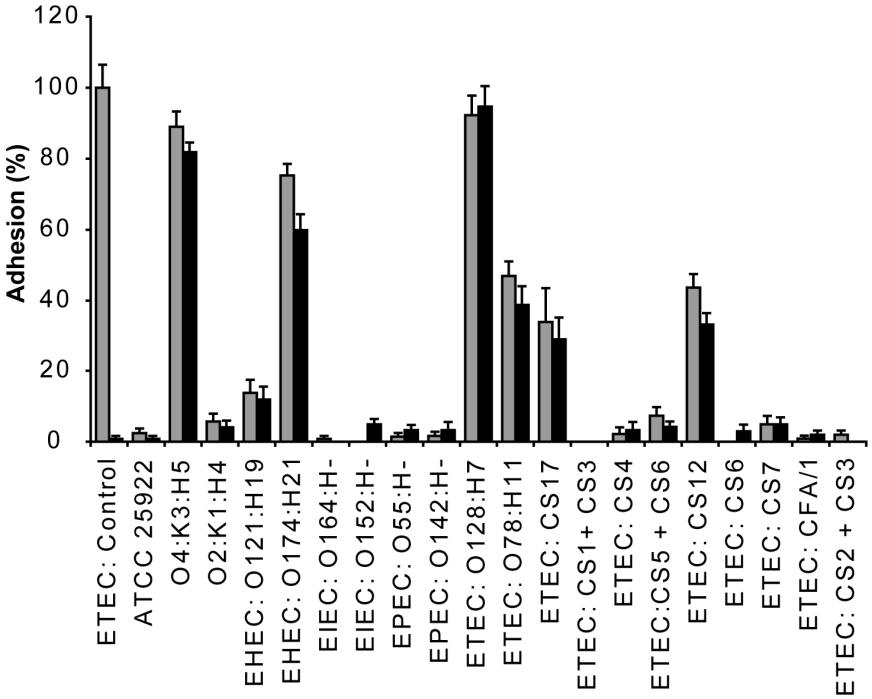

Fig. 6. Adhesion of different human *E. coli* strains to piglet intestinal brush border cells (gray bars) and the adhesion of these strains with addition of tempe extracts (black bars). The strains were obtained from the following sources: ETEC: control: piglet K88-positive O149:K91 (ID 1000), ID-Lelystad, 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.

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 7.

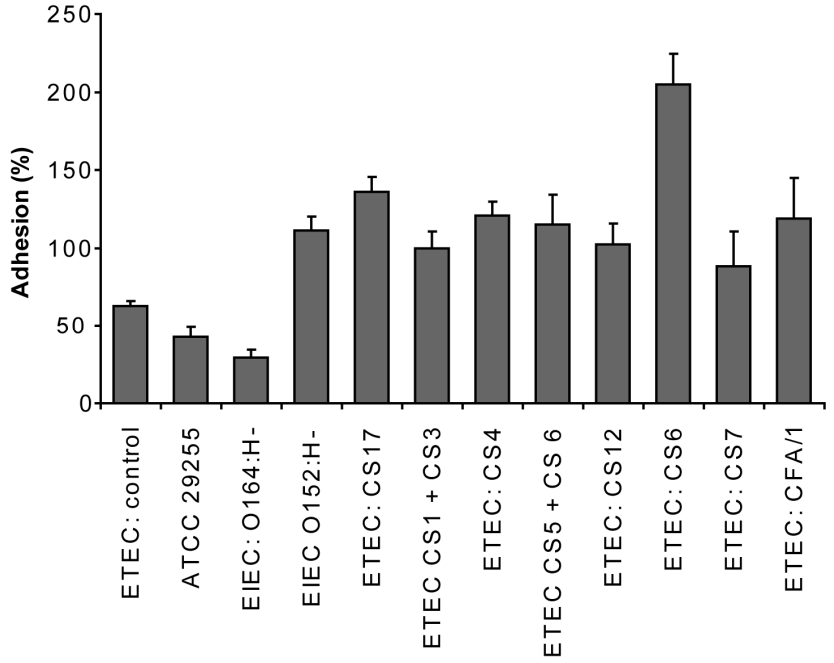

Fig. 7. Adhesion of different *E. coli* strains to Caco-2 intestinal epithelial cells. Bars represent mean values, expressed as % adhesion compared to the adhesion of the strain without tempe addition. Error bars represent SEM. The strains were collected from the following sources, with in brackets the number of replicates tested: 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.

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 (see table 3) in several human studies with children suffering from diarrhoea (Kalavi *et al*., 1996; Karyadi & Lukito, 1996; Mahmud *et al*., 1985; 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 a potential impact on diarrhoeal episodes. The interactions between diarrhoea and malnutrition as a cause or an effect are well recognised (Gadewar & Fasano, 2005; Gracey, 1996). 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 intestines by specific interaction with the adhesion op pathogens to the intestinal cells in humans.

4. Conclusions and future perspectives

4.1 Conclusions

The fact that diarrhoea is a major health problem worldwide in children as well as in farm animals, underlines the importance of the search for anti-diarrhoeal agents and investigation of their mode of action.

Tempe was found to be bioactive in two ways towards diarrhoea-associated bacteria. On the one hand, tempe (and soya beans to a lesser extent) inhibit 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) 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(s) are of carbohydrate nature, and contain arabinose as an important monosaccharide constituent. The bioactive component(s) are supposed to originate from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of legumes.

The antibacterial activity of tempe is caused by a proteinaceous component, which is liberated during the fermentation of soya beans. The inactivation of bacterial cells and spores appears to be caused by permeabilization of the cytoplastic membrane.

4.2 Future perspectives

Further characterization of the anti-adhesion component is needed to fully understand the mechanism of action. A well purified and characterized bioactive component could be tested in animal and human studies to verify the *in vitro* results in *in vivo* situations. Further research is required to exploit potential application of the bioactive principle in food or feed matrices. Another important issue to be researched is the possibility to liberate the arabinose containing medium-weight polysaccharides through the addition of specific enzymes in stead of fermentation.

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

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