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

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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.

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1. 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 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 (Egounlety, 2001; Kiers et al., 2000a), 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

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(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.

Table 2
Microorganisms used for soya bean fermentation.

Strain no.[a]	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

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

2. Materials and methods

2.1. 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 37 °C overnight. The strains were provided by the collection of the Animal Science Group, Lelystad, Wageningen University and 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 30 °C. 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 30 °C 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 30 °C 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 30 °C. 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}.

Table 1
ETEC strains.

Strain no.[a]	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	

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

2.2. 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 use as substrates for fermentation. Substrates were soaked overnight in tap water at 30 °C. 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 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 *R. 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 × 90 × 45 mm) and incubated for 48 h at

30 °C. Cooked and fermented substrates were stored and used for analyses.

2.3. Soya bean fermentation with different microbial inoculants

Soya beans were soaked overnight in tap water at 4 °C (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 121 °C 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 30 °C.

2.4. 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 30 °C; MRS-plates were incubated anaerobically. The development of the mould mycelium and appearance of the tempe cakes after incubation were assessed visually.

2.5. 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 37 °C for 30 min with continuous shaking. After centrifugation (2600*g*; 15 min, 4 °C), 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 80 °C for 24 h, from which dissolved dry matter was calculated. The remaining 90 ml was freeze-dried and used for subsequent experiments.

2.6. 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.

2.7. 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}.

2.8. 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 piglet 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 ID 1084 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, 20 °C). 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 1000×). 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.

2.9. 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).

3. Results

3.1. 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 Fig. 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

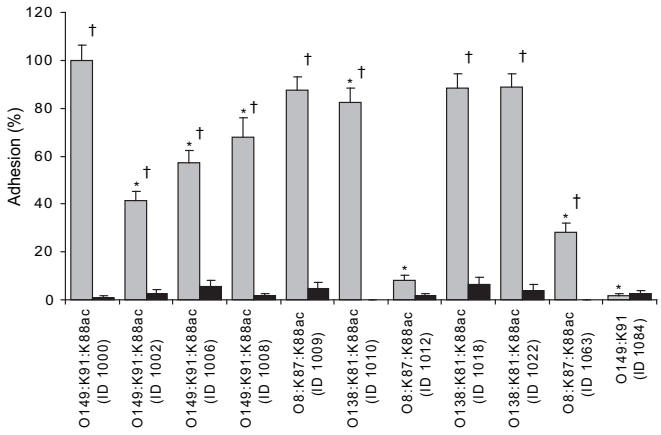

Fig. 1. Adhesion of different ETEC strains to piglet brush border cells. Gray bars represent adhesion without addition and black bars with addition of tempe extract (2.5 g l^{-1}). Bars represent mean values, expressed as % adhesion compared to the positive control (strain ID 1000) of 12 individual brush 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.

(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 (Fig. 1). The resulting adhesion values were of the same order as observed previously for strain ID 1000 (Roubos-van den Hil et al., 2009).

3.2. 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 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}.

3.3. Bioactivity of tempe prepared with different substrates

Bioactivity of the different cooked substrates are shown in Fig. 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

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[a] log CFU/g	TVC[b] 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

[a] LAB: Lactic Acid Bacteria.
[b] TVC: Total Viable Count of mesophilic aerobic bacteria.

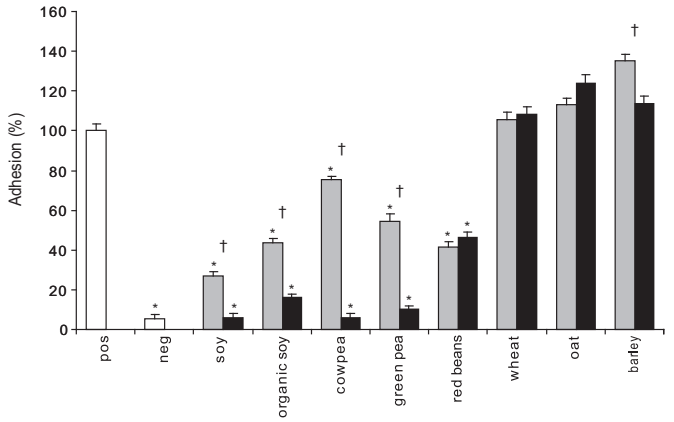

Fig. 2. Adhesion of ETEC K88 to brush border cells with addition of extracts of cooked and fermented substrates. White bars represent controls without any addition of extract; Gray bars represent extracts of cooked legumes and cereals (2.5 g l^{-1}); Black bars represent the fermented legumes and cereals (2.5 g l^{-1}). Bars represent 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.

not increased as a result of fermentation, which could very well be related to the incomplete fermentation of this substrate.

3.4. 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.

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.

3.5. Bioactivity of soya beans fermented with different microorganisms

The bioactivity of the fermented soya beans is shown in Fig. 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.

4. Discussion

Diarrhoeal disease in piglets is frequently due to infection by ETEC. It causes severe, watery diarrhoea especially in suckling and

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[a]	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[b]	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

[a] See Table 2 for names of microorganisms.
[b] ND, not determined.

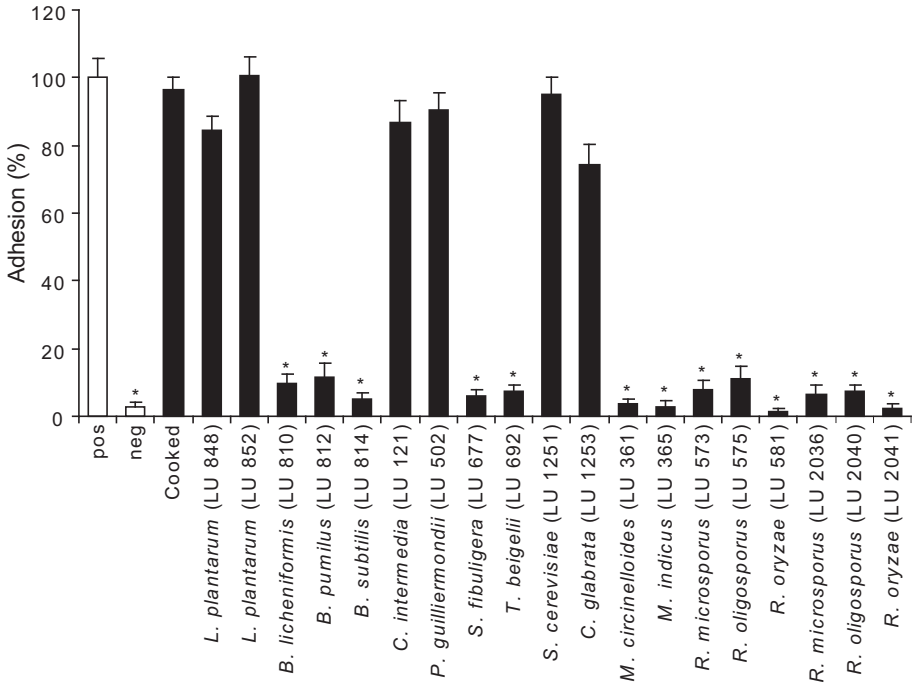

Fig. 3. Activity of soya beans after incubation for 48 h with different microorganisms. White bars represent controls without any addition of extract; Black bars represent extracts of soya beans incubated with different microorganism (1 g l^{-1}). Bars represent mean values, expressed as % adhesion compared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

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 diarrhoea 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 (Ashenafi and Busse, 1991; Nout et al., 1987). 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 (De Reu et al., 1995; Kiers et al., 2000a; Nout and Rombouts, 1990). 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 negligible, 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 (Ashenafi, 1994; Mulyowidarso et al., 1990; Samson et al., 1987) 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 (Nout and Kiers, 2005; Samson et al., 1987). In addition, tempe contains a range of bacteria such as LAB, *Bacillus* spp. and yeasts (Nout and Rombouts, 1990; Samson et al., 1987), 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 4 °C instead of 30 °C 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 (Kiers et al., 2000b; Nout et al., 1998; Sarkar et al., 1993). 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 yeasts species are specifically associated with tempe (Ashenafi and Busse, 1991; Feng et al., 2007b; Samson et al., 1987). 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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