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1 **Bioactivity of tempe by inhibiting adhesion of ETEC to intestinal**
2 **cells, as influenced by fermentation substrates and starter pure**
3 **cultures.**

4

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21

22 **Abstract**

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

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

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

37

38 **Keywords**

39 tempe, starter cultures, substrates, bioactivity, ETEC, adhesion

40

41

42 **1. Introduction**

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

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

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

65 Tempe is considered as the collective name for various pulses and cereals fermented with a
66 fungi belonging to the *Rhizopus* genus. Traditionally, tempe is made from soya beans and,

67 therefore, most research is done with soya bean tempe. However, other substrates such as
68 barley (Eklund-Jonsson et al. 2006; Feng et al. 2007a), chick pea (Ashenafi and Busse 1991),
69 cowpea (Egounlety 2001; Kiers et al. 2000a), groundbean (Egounlety 2001), horse bean
70 (Ashenafi and Busse 1991), pea (Ashenafi and Busse 1991), oats (Eklund-Jonsson et al.
71 2006), sorghum (Mugula and Lyimo 2000) and wheat (Hachmeister and Fung 1993) were
72 also reported to be suitable substrates to produce tempe. The impact of using different
73 substrates on the adhesion inhibition was not investigated before. Hence, the second aim of
74 this research was to determine the bioactivity of tempe prepared with different leguminous
75 and cereal substrates. Thereby several fermentation parameters were determined to confirm a
76 successful fermentation of the different products into a tempe product.

77 A diverse range of microorganisms may be encountered in tempe, including filamentous fungi
78 involved in the inoculation and fermentation of tempe, as well as high levels of bacteria and
79 yeasts (Nout and Rombouts 1990). Research on the microbial quality of commercial tempe in
80 The Netherlands showed that most samples had an aerobic plate count exceeding 10^7 CFU g^{-1} ,
81 with lactic acid bacteria over 10^7 CFU g^{-1} and yeast levels higher than 10^5 CFU g^{-1} in 69% of
82 the samples (Samson et al. 1987). While the contribution of these bacteria and yeasts to the
83 properties of tempe is only partly understood, they do play a role in flavour development and
84 chemical substrate modification (Nout and Rombouts 1990). Lactic acid bacteria were shown
85 to play a role in acidification of the soya beans during soaking, thereby preventing the growth
86 of spoilage causing microorganisms (Nout et al. 1987). In order to research the origin and
87 formation of the bioactive principle, the third aim of this research was to test the bioactivity of
88 soya beans inoculated with different microorganisms (isolated from tempe and similar
89 fermented products) and to monitor their growth during fermentation.

90

91

92 **2. Materials and methods**

93 **2.1 Micro-organisms**

94 Ten ETEC K88 strains and one ETEC K91 strain (for serotypes see table 1) were grown in
95 brain heart infusion (BHI) broth (Becton Dickinson, 237500) at 37°C overnight. The strains
96 were provided by the collection of the Animal Science Group, Lelystad, Wageningen
97 University and Research Centre, The Netherlands. The cultures were centrifuged (3000 g, 10
98 min) and washed twice with phosphate-buffered saline (PBS) (NaCl 136.89 mM, KCl 2.68
99 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 2.79 mM, pH 7.2), followed by suspending the pellets in
100 PBS, to an optical density of 0.75 corresponding with approximately 10⁹ CFU ml⁻¹.

101 For fermentation, 2 *Lactobacillus*, 8 mould, 3 *Bacillus* and 7 yeast strains from the Laboratory
102 of Food Microbiology, Wageningen University, Wageningen, The Netherlands were used
103 (table 2). *Lactobacillus* strains were maintained on de Man, Rogosa and Sharpe (MRS) agar
104 plates (Becton Dickinson, 288130). Two days before use in experiments the strains were
105 inoculated in MRS broth and incubated at 30°C. The cultures were then washed and diluted in
106 peptone physiological salt solution (PPS), containing neutralized bacteriological peptone 1 g
107 l⁻¹ (Oxoid, LP34) and NaCl 8.5 g l⁻¹, to approximately 10⁶ CFU ml⁻¹. *Bacillus* strains were
108 maintained on BHI agar. One day before the start of the experiment the strains were
109 inoculated in BHI broth and incubated at 30°C while shaking at 200 rpm. The cultures were
110 washed and diluted in PPS to approximately 10⁶ CFU ml⁻¹. Yeast strains were maintained on
111 Malt Extract Agar (MEA) (Oxoid, CM59). Two days before the start of the experiment strains
112 were inoculated in Malt Extract Broth (MEB) (Oxoid, CM57) and incubated at 30°C while
113 shaking at 200 rpm. The cultures were washed and diluted in PPS to approximately 10⁶ CFU
114 ml⁻¹. Moulds were maintained on MEA slopes; 7 days before the start of the experiment they
115 were inoculated on fresh MEA slopes and incubated at 30°C. A spore suspension was

116 prepared by adding 10 ml PPS per slope and releasing the sporangia to obtain a suspension
117 with a concentration of 10^5 - 10^6 CFU ml⁻¹.

118

119 **2.2 Tempe fermentation with different substrates and *Rhizopus* spp.**

120 Soya beans (yellow-skinned variety used for tempe making (USA), normal and organically
121 farmed), cowpea (Benin, West Africa), green pea, red bean, wheat, oat and barley (all from
122 local stores the Netherlands) were use as substrates for fermentation. Substrates were soaked
123 overnight in tap water at 30°C. In order to achieve an accelerated lactic acid fermentation
124 during this soaking step, the soak water had been inoculated with naturally acidified soaking
125 water (“backslop”) (Nout et al. 1987). Next, the substrates were rinsed with tap water and
126 cooked in fresh tap water for 20 min at a substrate:water ratio of 1:3 (w/v). Subsequently, the
127 substrates were cooled and surface dried at room temperature, and were spread out on mesh
128 trays for about 1 hour. For the fungal inoculation of the substrates a sporangiospore
129 suspension from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU 573) was
130 used. After inoculation with the spore suspension (10 ml kg⁻¹), the substrates (batches of 450
131 g) were packed into hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated for 48 h
132 at 30°C. Cooked and fermented substrates were stored and used for analyses.

133

134 **2.3 Soya bean fermentation with different microbial inoculants**

135 Soya beans were soaked overnight in tap water at 4°C (bean:water ratio 1:3 (w/v)) to avoid
136 fermentative acidification. Beans were washed and subsequently cooked for 20 minutes in
137 fresh tap water. After cooking, the beans were cooled and surface dried at room temperature.
138 Next, 100 g of beans were transferred into glass jars and sterilized at 121°C for 30 minutes.
139 After cooling to room temperature the beans were inoculated with 5 ml of the diluted

140 microbial cultures (table 2). After mixing, the beans were incubated aerobically for 48 h at
141 30°C.

142

143 **2.4 pH measurement and microbiological analyses**

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

154

155 **2.5 Dry matter content and solubility**

156 Dry matter content was determined by freeze drying 50 g of the samples. Freeze-dried
157 samples were ground to a fine flour (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan,
158 Germany) passing through a 0.5 mm sieve. Solubility was arbitrarily defined as the dissolving
159 capacity of 5 g sample in 100 ml water using subsequent extractions. It was quantified by
160 suspending 5 gram of freeze-dried sample in 50 ml distilled water and incubating at 37°C for
161 30 minutes with continuous shaking. After centrifugation (2600 g; 15 min, 4°C), supernatants
162 were collected and pellets were re-suspended with 20 ml of distilled water and centrifuged.
163 This re-suspension step was repeated once. The supernatants collected from the three
164 centrifugation steps were pooled and adjusted to 100 ml. Ten ml of this solution was oven-

165 dried at 80°C for 24 h, from which dissolved dry matter was calculated. The remaining 90 ml
166 was freeze-dried and used for subsequent experiments.

167

168 **2.6 Protein degradation**

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

179

180 **2.7 Reducing sugars**

181 Reducing sugars were determined according to Nelson-Somogyi (Green et al. 1989). Briefly,
182 1.0 g of freeze-dried ground samples was suspended in 25 ml distilled water with continuous
183 stirring for 1 h. Samples (450 µl) were mixed with 450 µl copper reagent, consisting of 4 parts
184 of K-Na-tartrate:Na₂CO₃:Na₂SO₄:NaHCO₃ (1:2:12:1.3) and 1 part of CuSO₄·5H₂O:Na₂SO₄
185 (1:9). The first reagent was prepared by boiling to completely dissolve the components. The
186 samples were cooked for 10 minutes and cooled to room temperature. Arsenomolybdate
187 reagent was prepared by mixing 26.5 g ammoniummolybdate in 450 ml distilled water with
188 addition of 21 ml concentrated H₂SO₄ and 3 g of Na₂HAsO₄·7H₂O in 25 ml distilled water
189 and this solution was incubated for 24h at 37°C. Prior to use, 1 part of this solution was mixed

190 with 2 parts of 1.5 M H₂SO₄ and 450µl of this reagent was added to the samples and mixed.
191 After 30 minutes 3 ml of distilled water was added and the absorption was measured at 520
192 nm. For the calibration glucose was used in a concentration of 0-150 µg ml⁻¹.

193

194 **2.8 Bioactivity assay**

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

202 Freeze dried soluble extracts (10 mg) were dissolved in 1 ml PBS, mixed in a head-over-tail
203 rotator for 1 h and centrifuged (10,000 g, 10 min, 20°C). Supernatants were diluted to
204 respectively 2.5 g l⁻¹ and 1 g l⁻¹, and 30 µl was mixed with 30 µl of ETEC K88 suspension
205 and 30 µl of brush border cell suspensions. The mixture was incubated at room temperature
206 with continuous gentle shaking (100 min⁻¹) in a plate shaker (Plate shaker KL2, Edmund
207 Bühler GmbH, Hechingen, Germany) for 1 h. The number of bacterial cells, adhering to 12
208 individual brush border cells, was determined by phase contrast microscopy (magnification
209 1000x). The proportion of adhesion was calculated as the average number of ETEC K88 per
210 brush border cell, relatively to the adhesion with the positive control.

211

212 **2.9 Statistical analyses**

213 The significance of the bioactivity experiments was evaluated by comparing means using one
214 or two-way ANOVA, followed by the Bonferonni post-test. Results were expressed as mean ±

215 SEM and differences were considered significant at $P < 0.05$. Statistics were performed with
216 Graphpad Prism version 4.03 for Windows (Graphpad Software, San Diego, CA, USA).

217

218 **3. Results**

219 **3.1 Bioactivity of soya bean tempe towards different ETEC bacteria**

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

233

234 **3.2 Monitoring tempe fermentation from different substrates**

235 After fermentation of different substrates with *Rhizopus microsporus* (LU 573), all substrates
236 except red beans and wheat were fully fermented, i.e. overgrown and penetrated by fungal
237 mycelium. The visual appearance of the fermented substrates was a dense cotton mycelium
238 that bound the individual legumes or cereals to a cake-like product. Also, the smell of these
239 products was fresh and typical of good quality tempe. In the red bean and wheat product the

240 mould had not fully penetrated the centre of the product, but mainly grown at the outside,
241 which resulted in a loose cake, which was easy to break.

242 Table 3 shows the fermentation parameters that were analyzed. During cooking the pH had
243 increased slightly (data not shown) and after 48 h of fermentation the pH was increased in all
244 of the fermented substrates except in oat. Solubility increased in all fermented substrates, with
245 a maximum of 7 times the initial amount of soluble material in fermented green peas. The
246 amount of free amino nitrogen was increased for all substrates during fermentation. In the
247 legumes the amount of free amino nitrogen before and after fermentation was higher than in
248 the cereals, but also the amount of proteins in the legume substrates was higher. All substrates
249 showed an increase of at least 2 times the amount of free amino nitrogen, except the wheat
250 and red bean tempe, which was in accordance with our expectations based on the poor
251 mycelium development in these products. The level of reducing sugars also increased strongly
252 during tempe fermentation, especially in the non-soya substrates.

253 Microbiological observations during the fermentation showed strong growth of LAB, up to
254 $\log 9 \text{ CFU g}^{-1}$, in the two soya products and the cowpea product. In the other substrates also
255 growth of LAB and total mesophilic aerobic bacteria was observed, but values were lower,
256 i.e. $\log 6-7 \text{ CFU g}^{-1}$.

257

258 **3.3 Bioactivity of tempe prepared with different substrates**

259 Bioactivity of the different cooked substrates are shown in figure 2. Whereas some significant
260 extent of adhesion inhibition was observed for the cooked legumes, the cooked cereal extracts
261 did not inhibit adherence significantly. After fermentation the bioactivity of the tempe
262 extracts increased significantly with all legume substrates, whereas the fermented cereals still
263 showed no activity. Soya, cowpea and green pea extracts inhibited the adhesion to values
264 lower than 20% of the positive control. The red beans substrate showed some adhesion

265 inhibition, but this activity was not increased as a result of fermentation, which could very
266 well be related to the incomplete fermentation of this substrate.

267

268 **3.4 Soya bean fermentation with different microorganisms**

269 Cooked and sterilized soya beans were inoculated with pure starter cultures of different
270 microorganisms, namely 2 *Lactobacillus* spp., 3 *Bacillus* spp., 6 yeasts and 8 mould strains
271 (table 2). After a 48 h incubation period, the fermented samples were analyzed and compared
272 with the cooked substrates, as shown in table 4.

273 The two tested LAB strains, that had been isolated from soya soaking water, grew to 9 log
274 CFU g⁻¹, a level that was also observed in regular tempe (table 3). During incubation the pH
275 decreased and a distinct sour odour was observed. All reducing sugars were utilized, but the
276 levels of free amino nitrogen did not change.

277 After incubation with the *Bacillus* spp., soya beans were sticky and an ammoniacal odour was
278 observed. The *Bacillus* spp. were able to grow up to 10 log CFU g⁻¹, while the pH and levels
279 of free amino nitrogen and reducing sugars had increased.

280 All yeasts were able to grow after inoculation to 8-9 log CFU g⁻¹. In contrast to the other
281 strains, the pH of soya beans incubated with *Saccharomyces cerevisiae* (LU 1251) and
282 *Candida glabrata* (LU 1253) was not increased during fermentation. *Saccharomycopsis*
283 *fibuligera* (LU 677) increased the levels of free amino nitrogen and reducing sugars. *Candida*
284 *intermedia* (LU 121), *Trichosporon beigelii* (LU 692) and *Saccharomyces cerevisiae* (LU
285 1251) caused an increase of reducing sugars. The other yeast strains (*Pichia guilliermondii*
286 LU 502 and *Candida glabrata* LU 1253) decreased the level of reducing sugars, but had no
287 effect on free amino nitrogen levels.

288 All mould strains were able to grow, and bound the soya beans together to a firm cake.
289 During fermentation all moulds caused an increase of reducing sugars and free amino
290 nitrogen levels.

291

292 **3.5 Bioactivity of soya beans fermented with different microorganisms**

293 The bioactivity of the fermented soya beans is shown in figure 3. All moulds and *Bacillus*
294 fermented soya beans showed adhesion inhibition of more than 90%. *Lactobacillus* fermented
295 soya beans caused no inhibition of adhesion. Of the yeast fermented soya beans only those
296 with *Saccharomycopsis fibuligera* (LU 677) and *Trichosporon beigeli* (LU 692) showed
297 inhibition of adhesion.

298

299 **4. Discussion**

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

308 During fermentation of soya beans with *Rhizopus* spp. diverse chemical modifications take
309 place. During the soaking stage the pH of the soaked substrates was lowered by LAB. A high
310 number of actively acidifying LAB mixed culture was obtained by using the back-slop
311 technique (data not shown). The soaking step is important for the quality of the tempe,
312 because it prevents the growth of spoilage causing bacteria (Ashenafi and Busse 1991; Nout

313 et al. 1987). During cooking of beans the pH will start to increase, which continues during
314 fermentation. This is a result of proteolysis and the release of ammonia following utilization
315 of amino acids as carbon and energy source by the mould (Sarkar et al. 1993). Oats did not
316 show a pH increase, which can be due to an initial pH decrease during the first hours of
317 fermentation during which sugars, and not proteins were used as substrates for growth. The
318 solubility of all substrates increased during fermentation, which is due to the enzymatic
319 degradation of macromolecules into substances of lower molecular weight with a higher
320 solubility (De Reu et al. 1995; Kiers et al. 2000a; Nout and Rombouts 1990). Enzymatic
321 degradation was also evidenced by the increased levels of free amino groups and reducing
322 sugars. Astuti (2000) showed that the effect of fermentation on total nitrogen content is
323 negligible, but increases of free amino acids take place during fermentation. Higher levels of
324 carbohydrates are found in the non-soya substrates, which upon degradation, result in higher
325 values of reducing sugars. Research conducted elsewhere (Ashenafi 1994; Mulyowidarso et
326 al. 1990; Samson et al. 1987) indicated that in tempe total bacterial counts can reach 10^9 CFU
327 g^{-1} and LAB can reach levels of 10^8 - 10^9 CFU g^{-1} , which is comparable with our observations
328 in soya and cowpea tempe. The other substrates also supported microbial growth, but
329 remarkably less, for example in barley tempe the counts were comparable with data reported
330 by Feng et al. (2005). Different levels of growth achieved in diverse substrates are assumedly
331 associated with their individual nutrient composition.

332 Bioactivity was measured in all extracts of leguminous tempe and this activity increased
333 during fermentation. In contrast, the cereal-derived tempe products showed no bioactivity at
334 all. It appears that during fermentation an active component was released or formed by
335 enzymatic breakdown from leguminous substrates. Mould biomass itself has no bioactivity
336 since well-grown cereal-derived tempe lacked inhibition activity. The active component(s) is,
337 therefore, specific for legumes. This could be related to the higher protein content or the

338 protein composition in legume seeds. Another remarkable difference is the relatively high
339 amount of isoflavones present in legumes, which are not (or at very low levels) present in
340 cereals (Liggins et al. 2002). Also the cell wall is different, monocotyledons (cereals) contain
341 cellulose fibrils in close association with arabinoxylans, whereas dicotyledons (legumes)
342 contain cellulose with pectin and xyloglucans (Harris and Smith 2006).

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

348 Before inoculation with the pure microbial strains the soya beans were soaked overnight at
349 4°C instead of 30°C to avoid fermentative acidification, since this acidification could
350 influence the growth of acid sensitive strains. When testing the effect of tempe-derived pure
351 microbial strains on soya beans, we observed that *Lactobacillus* spp. assimilated all available
352 sugars to form lactic acid as indicated by concomitant pH decrease. The modification of soya
353 beans with *Bacillus* spp. can be compared with that during the fermentation of *Kinema*, a
354 traditional *Bacillus* fermented soya bean food in India and Nepal. These fermentations are
355 characterized by extensive hydrolysis of proteins into amino acids, peptides and ammonia and
356 a typical sticky appearance of the soya beans (Kiers et al. 2000b; Nout et al. 1998; Sarkar et
357 al. 1993). Our observations of *Bacillus* fermented soya beans were similar to *Kinema*. The
358 occurrence of yeasts has been reported in tempe products as yeasts can grow well in mixed
359 microflora with lactic acid bacteria and filamentous fungi, but no yeasts species are
360 specifically associated with tempe (Ashenafi and Busse 1991; Feng et al. 2007b; Samson et
361 al. 1987). Our observations show that yeasts found in tempe were able to grow on soya beans
362 and some yeasts were also able to interact (by degrading macromolecules) with the soya

363 beans. The soya beans fermented with the different mould strains were similar to tempe
364 prepared following the usual process, confirming that tempe can be made without co-
365 inoculants.

366 All *Bacillus* spp., yeast strains LU 677 and LU 692 and all tested mould strains caused
367 inhibition of the ETEC adhesion after incubation with soya beans. Thus, activity was not
368 related to a specific microorganism, but instead the degradation of certain macromolecules is
369 needed to release or form bioactive component(s) from the soya beans.

370 In conclusion, tempe derived from leguminous seeds is bioactive, i.e. reduces adhesion of
371 ETEC to piglet brush border cells, whereas tempe derived from cereals is inactive. The
372 bioactive component(s) is released or formed during fermentation from leguminous matter.
373 The capability to release or form bioactive component(s) is not specific for one microbial
374 species. A range of ETEC strains was shown to be sensitive for the bioactive component,
375 making this bioactivity of potential interest for application in animal husbandry.

376 Further research to elucidate the nature of the bioactive component in fermented leguminous
377 seeds will be required.

378

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383

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460 enteropathogenic *Escherichia coli* to pig intestinal brush borders: the existence of two
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464

465 **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	

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467 ^a Strains were obtained from the collection of the Animal Sciences Group, Wageningen

468 University and Research Centre, Lelystad, The Netherlands.

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477 **Table 2:** Microorganisms used for soya bean fermentation

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

478

479 ^a Strains were obtained from the Laboratory of Food Microbiology, Wageningen University,

480 Wageningen, The Netherlands

481

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

		pH	solubility (g/ 100g dry matter)	Amino nitrogen (mmol free amino group/ 100g dry matter)	protein (g/100g dry matter)	Reducing sugars (mg/100g 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 (48h)	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 (48h)	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 (48h)	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 (48h)	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 (48h)	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 (48h)	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 (48h)	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 (48h)	5.3	9.0	8.1	11.2	887.5	6.3	7.6

484

485 ^a LAB: Lactic Acid Bacteria

486 ^b TVC: Total Viable Count of mesophilic aerobic bacteria.

487

488

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

Sample type	pH	Amino nitrogen (mmol free amino group/ 100g dry matter)	Reducing sugars (mg/100g dry matter)	Inoculation (log CFU/g)	Incubated 48h (log CFU/g)
Cooked	6.6	19.9	59.2	-	-
<i>Lactobacillus</i>					
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
<i>Bacillus</i>					
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

491

492 ^a See Table 2 for names of microorganisms

493 ^b ND, not determined

494

495 **Legends to figures**

496

497 **Figure 1: Adhesion of different ETEC strains to piglet brush border cells.**

498 Gray bars represent adhesion without addition and black bars with addition of tempe extract
499 (2.5 g l⁻¹). Bars represent mean values, expressed as % adhesion compared to the positive
500 control (strain ID 1000) of 12 individual brush borders. Error bars represent SEM. Bars with
501 asterisk (*) differ significantly from strain ID 1000. Bars with † represent a significant effect
502 of addition of tempe extract.

503

504 **Figure 2: Adhesion of ETEC K88 to brush border cells with addition of extracts of**
505 **cooked and fermented substrates.**

506 White bars represent controls without any addition of extract; Grey bars represent extracts of
507 cooked legumes and cereals (2.5 g l⁻¹); Black bars represent the fermented legumes and
508 cereals (2.5 g l⁻¹). Bars represents mean values, expressed as % adhesion compared to the
509 positive control of 12 individual brush borders. Error bars represent SEM. Bars with asterisk
510 (*) inhibit adhesion significantly compared with the positive control. Bars with † represent a
511 significant difference between cooked and fermented substrates.

512

513 **Figure 3: Activity of soya beans after incubation for 48 h with different microorganisms.**

514 White bars represent controls without any addition of extract; Black bars represent extracts of
515 soya beans incubated with different microorganism (1 g l⁻¹). Bars represent mean values,
516 expressed as % adhesion compared to the positive control of 12 measurements. Error bars
517 represent SEM. Bars with asterisk (*) inhibit adhesion significantly compared with the
518 positive control.

519

Figure 1

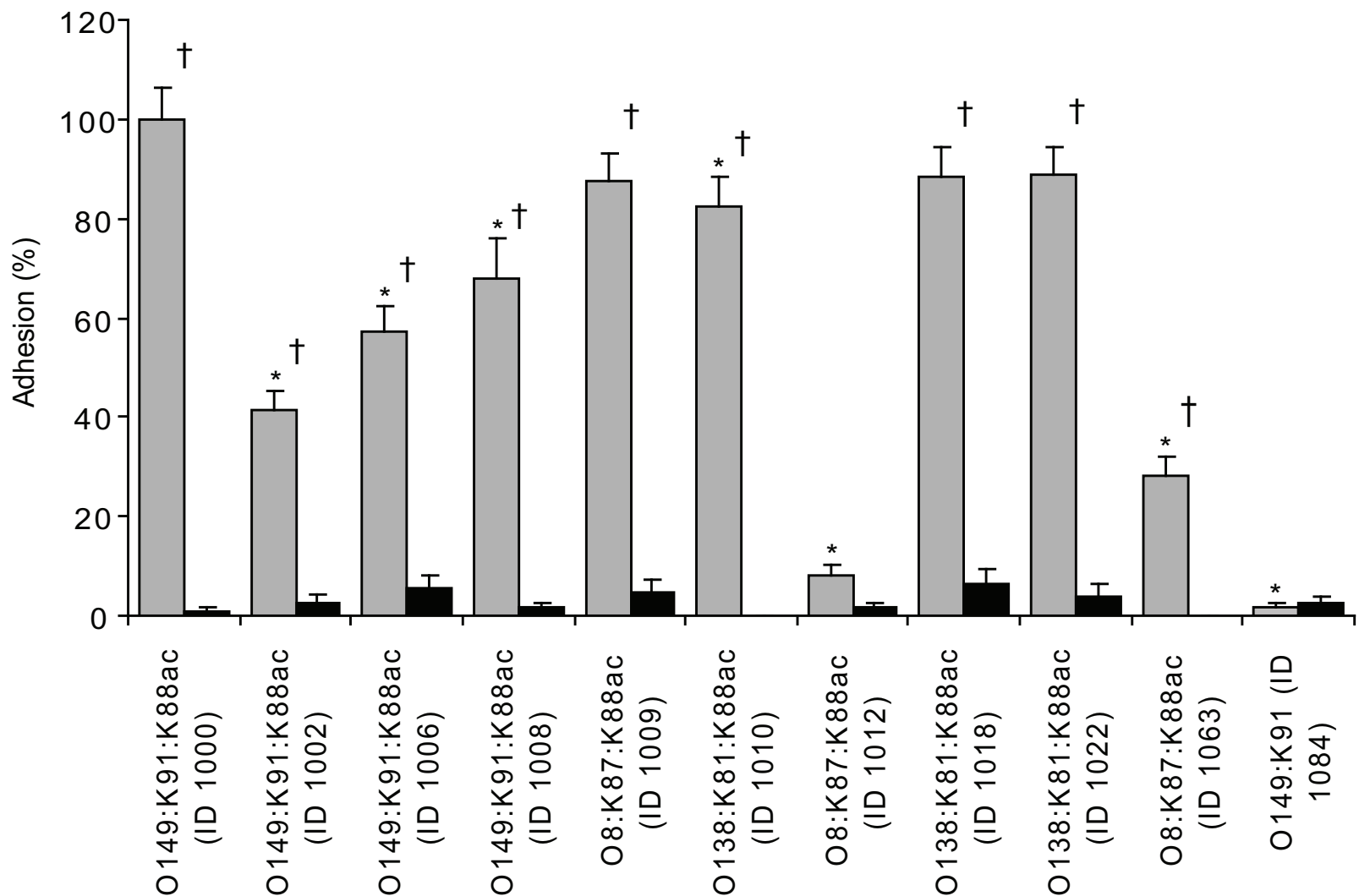


Figure 2

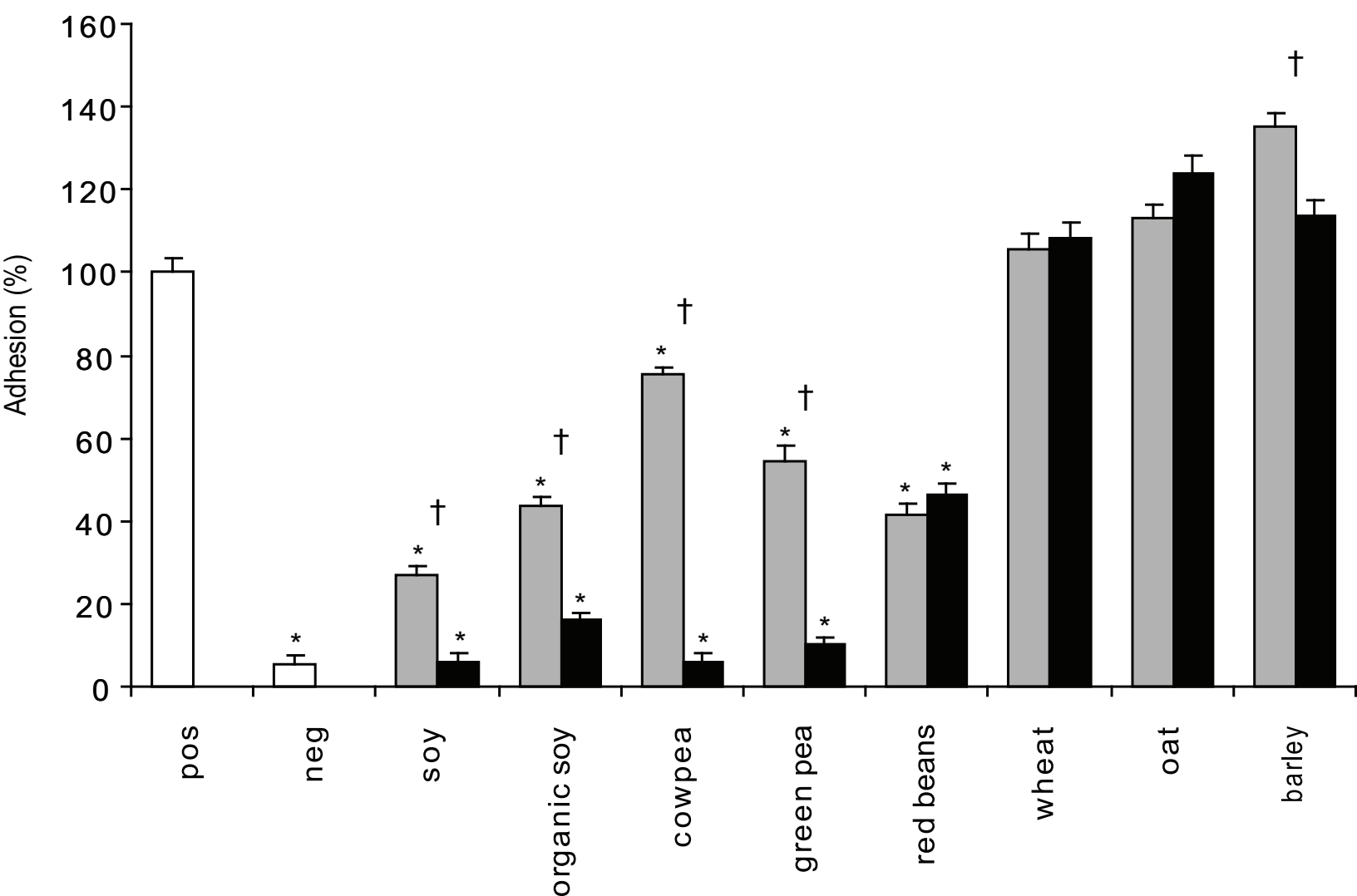


Figure 3

