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

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Abstract: Tropical legumes, ie soya bean and cowpea, were pre-treated and subsequently fermented using pure cultures of *Rhizopus* spp. Impact of soaking, cooking and fermentation of the legumes on their digestibility was determined using an *in vitro* digestion method. Processing of white maize included, amongst others, natural lactic acid fermentation, cooking and saccharification using barley malt. An *in vitro* method was standardised to carry out comparative determinations of the dry matter digestibility of cereal and legume food samples as a function of processing conditions, without attempting to exactly mimic gastrointestinal digestion. Using this method based on upper digestive tract digestion, it was observed that digestibility of the legumes increased during cooking and fermentation. Cooking improved the total digestibility of both soya bean and cowpea from 36.5 to 44.8% and from 15.4 to 40.9% respectively. Subsequent fungal fermentation increased total digestibility only by about 3% for both soya bean and cowpea. Digestibility was also influenced by fungal strain and fermentation time. Cooking and subsequent saccharification using malt almost tripled total digestibility of white maize from 25.5 to 63.6%, whereas lactic fermentation of maize had no effect on *in vitro* dry matter digestibility. Although total digestibility of cooked legumes was only slightly improved by mould fermentation (3% for both soya bean and cowpea), the level of water-soluble dry matter of food samples increased during fermentation with *Rhizopus oryzae* from 7.0 up to 27.3% for soya bean and from 4.3 up to 24.1% for cowpea. These fermented products could therefore play a role as sources of easily available nutrients for individuals suffering from digestive disorders.

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Keywords: *in vitro* digestibility; soya bean; cowpea; maize; processing; *Rhizopus*; tempe

INTRODUCTION

Legumes and cereals are rich in nutrients and bioactive substances, but they also contain various indigestible constituents such as non-starch polysaccharides, as well as antinutritional factors (ANFs) such as trypsin inhibitors and phytic acid. Various processing methods such as soaking, dehulling, germination,^{1,2} cooking,³ extrusion^{4,5} and fermentation^{6–10} have been reported to achieve improvements in nutritional value and digestibility.

Tempe is a traditional Indonesian fungal fermented food made from dehulled, soaked and cooked soya beans inoculated with *Rhizopus* sp. Tempe has high levels of protein and unsaturated lipids, has a pleasant flavour and texture and has been reported to be easily digestible.^{6–8} The digestibility of tempe might be caused by enzymatic degradation of soya bean polymeric substances resulting in soluble solids, particularly soluble nitrogenous compounds, by the mould during fermentation.⁸ Occasionally other legumes or cereals are used for tempe fermentation.^{7,8,11} Kalavi *et al*¹² reported a significantly higher growth rate, shorter duration of diarrhoeal episodes and shorter rehabilita-

tion period in a group of children suffering from protein energy malnutrition supplemented with a porridge containing tempe and yellow maize, compared to a similar porridge made of milk and yellow maize.

In vitro digestibility studies have been carried out previously. The methods were designed to monitor specific compounds such as water-soluble dry matter, starch, protein^{13,14} or non-digestible materials.¹⁵ Alternative methods were developed aimed at simulating the complete human gastrointestinal tract.¹⁶

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

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MATERIALS AND METHODS

Micro-organisms

Rhizopus microsporus var *oligosporus* LU 575 (NRRL 5905) and *Rhizopus oryzae* LU 582 were grown on malt extract agar (Oxoid, CM 59). Sporangiospore suspensions were obtained by scraping off the sporangia from a slant culture after 7 days incubation at 30 °C and suspending them in sterile distilled water with 0.85% NaCl and 0.1% peptone. The viable count varied between 10^5 and 10^6 colony-forming units (cfu) ml⁻¹ when determined on Rose-Bengal Chloramphenicol Agar (Oxoid, CM 549).

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

Processing of soya bean and cowpea

Dehulled yellow-seeded soya bean (*Glycine max*) and non-dehulled cowpea (*Vigna unguiculata*) were soaked overnight in tapwater during three cycles of accelerated acidification.¹⁷ Subsequently the beans and peas were washed with tapwater, cooked in fresh tapwater for 20 min (ratio beans/water of 1:3), cooled and superficially dried at room temperature. At this stage the cooked cowpeas were mildly squeezed to release the hulls, yielding a mixture of cooked cotyledons and hulls. After inoculation with the sporangiospore suspension (1% v/w) the beans and peas (450 g) were packed into hard-plastic, perforated boxes ($205 \times 90 \times 45$ mm⁻¹) and incubated at 30 °C for 44 h.

Processing of maize

Raw white maize (*Zea mays*) was coarsely ground to a flour (Fritsch, type Pulverisette 14, Germany). Two

optional processing schemes A and B were followed. Option A started with accelerated natural lactic fermentation (fermentation cycles of 24 h at 30 °C) of a mixture of flour and tapwater in a ratio of 40 g flour to 60 g tapwater. Tapwater was added to the acidified dough to adjust dry matter to 15% and the porridge was cooked for 10 min.¹⁸ Processing option B started with cooking a mixture of raw maize flour and tapwater (15% dry matter) for 10 min, followed by cooling to 50–55 °C and addition of 1% w/w of malted barley, maintaining the temperature at 50–55 °C for 30 min. After saccharification the product was autoclaved for 30 min at 121 °C. Fermentation of the autoclaved product was carried out by inoculating the sterile porridge with a pure culture of *Lactobacillus* sp at 10^3 cfu g⁻¹ and incubating for 24 h at 30 °C.

Sample preparation

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

In vitro digestion

The *in vitro* digestion method consisted of two steps as outlined below and illustrated in Fig 1. The method was carried out with 5 g of non-defatted sample as well as with the residue obtained after defatting a 5 g sample.

Step 1. Enzymatic degradation

Samples (X) were suspended in distilled water (30 ml). The samples were incubated while stirring with an

Figure 1. Flowsheet of the *in vitro* digestion method. All parameters are in g: X, sample; P, pellet after degradation; Px, part of X ending up in P; Pb, part of reagent blank ending up in P; S, supernatant after degradation; Sx, part of X ending up in S; Sb, part of reagent blank ending up in S; HCl, amount of HCl added; NaHCO₃, amount of NaHCO₃ added; Pu, pellet after solubilisation; Su, supernatant after solubilisation; D, dialysate of S; Dx, part of X ending up in D; Db, part of reagent blank ending up in D; Du, dialysate of Su; R, retentate of S; Rx, part of X ending up in R; Rb, part of reagent blank ending up in R; Ru, retentate of Su.

α -amylase solution (2 ml) consisting of 12 500 units l^{-1} α -amylase (Sigma A-1031), 1.5 $g l^{-1}$ NaCl, 1.5 $g l^{-1}$ K_2HPO_4 and 0.5 $g l^{-1}$ Na_2CO_3 (pH 7.0) for 30 min at 37 °C. Next the pH was adjusted to 4.0 using 5 M HCl and the suspensions were incubated with 8 ml of stomach medium (0.1 $g l^{-1}$ lipase (Amano Pharmaceuticals, *Rhizopus* F-AP15), 0.125 $g l^{-1}$ pepsin (Sigma P-6887), 3.1 $g l^{-1}$ NaCl, 1.1 $g l^{-1}$ KCl, 0.6 $g l^{-1}$ Na_2CO_3 , 0.11 $g l^{-1}$ $CaCl_2$, pH 4.0) for 1 h at 37 °C. The pH was then adjusted to 6.0 using solid $NaHCO_3$. Finally, 10 ml of a 2% pancreatic solution (20.0 $g l^{-1}$ pancreatin (Sigma P-1750), 5.0 $g l^{-1}$ bile (Sigma B-3883), 5.0 $g l^{-1}$ NaCl, 0.68 $g l^{-1}$ KH_2PO_4 , 0.3 $g l^{-1}$ Na_2HPO_4 , 0.84 $g l^{-1}$ $NaHCO_3$) was added and the suspensions were incubated for 30 min at 37 °C. Subsequently the suspensions were centrifuged at 3000 $\times g$ for 15 min at 4 °C. The supernatant was decanted and the pellet was washed twice in 20 ml of distilled water. Supernatants obtained were pooled and aliquots were taken for dry matter content determination by drying for 24 h at 80 °C in triplicate, resulting in S (g). The pellet was freeze-dried to determine its dry weight P (g).

Step 2. Dialysis

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

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

Corrections and blank

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

Solubility and absorbability without enzymatic treatment

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

Calculations

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

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

Coefficients of variation

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

RESULTS

pH changes during processing

Changes in pH during processing of soya bean and cowpea are shown in Table 1. Soaking with the accelerated acidification technique gave a pH of 4.2 and 4.1 for soya bean and cowpea respectively. Cooking resulted in an increase in pH to 4.9 for both substrates. Fungal fermentation generally resulted in pH increase as illustrated by maximum pH levels of 6.7 and 5.8 obtained in soya bean and cowpea fermented with *Rhizopus oryzae* LU 582 and *Rhizopus oligosporus* LU 575 respectively. Although not necessarily correlated, these pH changes were in accordance with comparative fungal growth activities that were measured by evolution of metabolic heat (data not shown).

Accelerated natural fermentation of maize by back-slopping resulted in a pH of 3.7 (Table 2). Using a starter culture of *Lactobacillus* sp, a similar pH decrease was seen after fermentation of the autoclaved material for 24 h at 30 °C (final $\log_{10}$ cfu g^{-1} = 10.4).

Crude lipid content

Percentages of crude lipid content on a dry weight basis are shown in Tables 1 and 2. Due to soaking and

Table 1. Characteristics of processed and fermented soya bean and cowpea. Data represent single determinations

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

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

Table 2. Characteristics of processed and fermented maize

	pH	Crude lipid (% dry matter)	Solubility (% fat-free dry matter)
R ^a	5.6	4.0	5.6
S	3.7	4.0	4.6
SC	3.8	2.8	5.4
C	5.7	2.8	6.1
M	5.8	3.4	73.6
CMA	5.9	3.4	70.9
CMAL	3.8	3.6	68.9

^a See Table 1; M, malted; A, autoclaved; L, fermented with *Lactobacillus* sp.

cooking of soya bean, the crude lipid content increased markedly. Fermentation of soya bean with LU 575 slightly increased the crude lipid content to 29.5%, and with LU 582 it decreased to 25.9%. Fermentation of cowpea with these moulds appeared to increase the total crude lipid content, even up to 4.8% for LU 582.

Solubility

Solubility was reduced markedly in soaked and cooked soya beans and cowpeas (Table 1). During subsequent fermentation of soya bean and cowpea, solubility increased up to two to six times compared to the cooked samples. LU 582 resulted in higher solubility than LU 575.

Solubility of maize was hardly affected by cooking and fermentation (Table 2). Malting of cooked maize, however, resulted in a 12-fold increased solubility.

Absorbability and digestibility

In Table 3, absorbability and digestibility of processed and fermented soya bean, cowpea and maize are shown. For soya bean and cowpea, both absorbability and digestibility decreased after soaking. However, in cooked samples, digestibility was increased whereas absorbability continued to decrease. Fermentation of soya bean resulted in considerable increases in absorbability, but digestibility only increased slightly. For cowpea this was even more pronounced. Whereas cooking of maize resulted in high digestibility, absorbability did not increase until saccharification of cooked maize was conducted.

Total digestibility of defatted samples

In the processed soya bean samples the crude lipid contributed most to the total digestibility (Tables 1 and 4). The highest contribution to the total digestibility of cowpea samples was derived from the digestibility (absorbable material after enzymatic degradation) component.

The decrease in total digestibility due to soaking was followed by a major increase due to cooking. During soya bean fermentation with LU 575, total digestibility

Table 3. Absorbability, digestibility and part of the digestible material that was already absorbable before enzyme treatment expressed as the ratio between absorbability and digestibility (A/D), for processed and fermented soya bean, cowpea and maize. Digestibility represent the average of duplicate analysis

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

^a See Tables 1 and 2.

Table 4. Total digestibility (% dry matter) of defatted and non-defatted soya bean, cowpea and maize^a

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

^a Duplicate values.^b See Tables 1 and 2.

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

Total digestibility of processed maize is shown in Table 4. Cooking resulted in the biggest increase in total digestibility, but subsequent saccharification using malt also significantly improved total digestibility. Fermentation had only minor effects on total digestibility.

In vitro digestion of defatted vs non-defatted samples

In Table 4, total digestibility of defatted and non-defatted samples is shown. The data show that the values for defatted soya bean samples are much higher than for non-defatted samples. In the case of cowpea and maize, values for total digestibility of defatted and non-defatted samples are closer.

DISCUSSION

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

at low crude lipid contents estimations of dry matter losses are less accurate.

Although the dry matter losses might seem a waste, soaking is essential to increase the moisture content and to leach out several ANFs, and cooking is essential to destroy potential pathogenic bacteria, to inactivate trypsin inhibitors and to release some of the nutrients essential for mould growth.⁶

Acidification during the soaking of maize flour resulted in a sufficient pH decrease to contribute significantly to food safety. Upon fermentation of maize and other cereals, nutritional benefits such as degradation of ANFs, increased mineral bioavailability, improvement in protein digestibility and degradation of flatulence-causing oligosaccharides may result.⁹

Tempe fermentation of soya bean and cowpea

During tempe fermentation the fermenting micro-organisms, mainly *Rhizopus* spp, induce many compositional changes, including degradation of protein, lipid and carbohydrates, catalysed by a variety of fungal proteases, lipases and carbohydrases.¹⁹ In addition, a decrease in ANFs is associated with the action of the moulds and their enzymes.²⁰

Generally a pH increase is observed resulting from protein degradation and liberation of ammonia.⁶ However, there are significant differences between *Rhizopus* spp and between the substrates in regard to pH changes and substrate modifications.²¹ This is also shown by our present data.

Changes in crude lipid content during fermentation might result from metabolic activity such as assimilation and synthesis, as well as from changes in non-lipid dry matter.^{19,22}

Solubility

The less pronounced decrease in solubility in the case of cowpea can be attributed to the presence of the cowpea seed coat acting as a barrier to diffusion.

A marked increase in water-soluble compounds as was shown after fermentation using *Rhizopus* spp has been reported elsewhere for tempe fermentation.²³

The high increase in solubility due to the saccharification of cooked maize can be explained mainly by

the transformation of gelatinised starch into water-soluble dextrans, oligosaccharides, maltose and glucose.

Absorbability

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

Total digestibility

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

The big increase in total digestibility of maize and cowpea is probably due to the gelatinisation of the starch present in large amounts in these materials, making it more readily available for the enzymatic degradation. Processing of cowpea is essential because ANFs in raw cowpea could result in pathophysiological changes in gut morphology leading to impaired absorption of nutrients and consequently a diminished growth of pig weaning pigs.²⁴

Although tempe fermentation did not increase the total digestibility of soya bean and cowpea to a large extent, it was shown that the amount of absorbable matter without enzymatic degradation (absorbability) increased markedly owing to mould fermentation. Digestibility of fermented legumes was only slightly improved. From the ratio between absorbability and digestibility it is also shown that mould fermentation contributed up to 50–60% of the digestibility. From this it can be concluded that mould fermentation already 'pre-digested' the material to a significant extent. Similarly, after saccharification of cooked maize with malt, hardly any additional absorbable material is produced after incubation with gastrointestinal enzymes. Saccharification using malt was confirmed to be a suitable technique for the preparation of highly digestible, nutrient-dense cereal porridges.²⁵ These findings have particular relevance for their use in formulated foods for individuals suffering from a malfunctioning gastrointestinal physiology.

In vivo trials using rats and neonatal pigs showed only minor differences in growth, daily weight gain and protein efficiency when fermented soya beans and cowpeas were fed compared to unfermented controls.^{6,7,26–28} This could lead to the conclusion that fermentation of legumes using *Rhizopus* spp would hardly improve their *in vivo* digestibility in healthy rats and piglets. However, the small increase in *in vitro* digestibility and, even more, the large increase in absorbability caused by fermentation observed in this

study could have beneficial physiological effects in the case of malfunctioning gastrointestinal digestive systems. These foods are therefore expected to offer benefits when feeding weaning pigs, or children suffering from malnutrition and/or acute diarrhoea for whom the need for easily digestible rehabilitation foods is high. The use of tempe in rehabilitation of children suffering from protein–energy malnutrition in Indonesia has already been reported by Karyadi *et al*,²⁹ who showed a greater nutritional impact of food mixtures containing tempe than of those containing cooked soya beans.

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