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Analytical Methods

Effect of soybean processing on content and bioaccessibility of folate, vitamin B12 and isoflavones in tofu and tempe

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ABSTRACT

Purpose: To compare the content of bioaccessible folate, vitamin B12, and isoflavones in tofu and tempe, as influenced by soybean variety and food processing, particularly fermentation.

Principal results: Raw soybeans contained 2207–2671 µg/kg (dry matter) folate, cooked tempe 1493–4143, and cooked tofu 968–1273 µg/kg, the difference was attributed to the fermentation in tempe. Vitamin B12 was detected only in tempe (0.16–0.72 µg/kg). Isoflavone aglycones were formed during soaking of soybeans, with only minor differences between the contents in cooked tempe (average 1922–2968 µg/kg) or tofu (1667–2782 µg/kg) but strongly depending on bean variety.

Conclusions: Folate and vitamin B12 contents were mainly influenced by microbial activity during fermentation, whereas isoflavone aglycone content was determined by bean variety. Tofu had lower folate and vitamin B12, but equal isoflavone contents as tempe. Bioaccessibility of folate (80–100%) and isoflavone aglycones (100%) were high.

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1. Introduction

Of the many uses of soybeans, the Asian vegetarian products tofu (Rekha & Vijayalakshmi, 2011) and tempe or tempeh (Nout & Kiers, 2005), enjoy an increasing consumer interest (Forbes, Erdman, Parker, Kondo, & Ketelsen, 1983; Kasaoka, Astuti, Uehara, Suzuki, & Goto, 1997; Poneris & Erdman, 1988; Stodolak & Starzynska-Janiszewska, 2008; Weaver et al., 2002; Yan, Graef, Reeves, & Johnson, 2009) because of their taste, easy digestibility, and their association with health. Tofu is made by soaking soybeans in water, wet-milling, removal of soy fibre (named okara) by filtration, cooking and coagulation of the soy protein by the addition of calcium salts. The precipitated protein is collected, and pressed to firm consistency. Tempe is made by soaking soybeans in water, removal of the seed coat, boiling the cotyledons, cooling and inoculating them with sporangiospores of pure or undefined traditional mixtures (Nout, Martoyuwono, Bonné, & Odamtten, 1992) of varieties of the fungus *Rhizopus microsporus*, and incubating as a solid bed at 30–37 °C for 40–48 h. During soybean soaking prior to fungal fermentation, also a bacterial

fermentation may occur (Nout & Kiers, 2005). Tofu and tempe are both cooked prior to consumption. Fermented soybean foods contain several health-beneficial bio-active compounds (Murooka & Yamshita, 2008), of which folate, vitamin B12, and isoflavones are prominent.

Folate (also known as vitamin B9) is a generic term referring to various derivatives, vitamers, of folic acid. Adequate folate intake is known to prevent neural tube defects and megaloblastic anaemia. Furthermore, folate is intensively studied for its role in decreasing the risk of cardiovascular disease, several cancer types, and cognitive disorders (Cuppen & Bolander-Gouaille, 2005; Luccock, 2000). Folate intake in countries without mandatory fortification generally does not reach the recommended daily intake. Thus, it is important to study dietary folate sources and ways to naturally enhance folate intake. Tofu has been recognised as a good source of folate (1084 µg/kg dry matter (DM) (Ginting, Arcot, & Cox, 2003)). Folate synthesis is also associated with fungal (Sanke, Miyamoto, & Murata, 1971) metabolism. In tempe, folate values as high as 2980 and 4164 µg/kg DM have been reported (Arcot, Wong, & Shrestha, 2002; Ginting & Arcot, 2004). Assuming a folate content of 1500 µg/kg fresh weight, 100 g of tempe would provide approximately one third of the recommended nutrient intake of folate (400 µg/day; (FAO/WHO., 2004)) provided that all folate is bioavailable, i.e.

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absorbed and metabolised. Bioavailability is often determined in human intervention studies which are time and cost consuming. An efficient alternative is to measure bioaccessibility in a dynamic *in vitro* gastrointestinal model which is representative for *in vivo* bioavailability in humans (Verwei, Arkbåge et al., 2005; Verwei, van den Berg, Havenaar, & Groten, 2005), assuming complete absorption (Verwei, Freidig, Havenaar, & Groten, 2006).

Vitamin B12 represents corrinoids exhibiting the biological activity of cyanocobalamin. Its synthesis is restricted only to some bacteria and archaea (Raux, Schubert, & Warren, 2000). Thus, foods of plant origin do not contain vitamin B12 unless fermented or contaminated. Vitamin B12 deficiency causes disturbances in cell division and leads to similar haematological changes as folate deficiency but in addition to megaloblastic anaemia, a disease of the nervous system, neuropathy, may occur (Truswell, 2007). Due to cobalamin stores in the body, dietary vitamin B12 deficiency is rare and develops very slowly. However, deficiency may be a significant risk for people on a strict vegan diet and among elderly (Stabler & Allen, 2004). Whereas tofu is not supposed to contain detectable vitamin B12, for tempe vitamin B12 contents from 4 to 130 µg/kg DM have been reported (Areekul et al., 1990; Keuth & Bisping, 1993; Liem, Steinkraus, & Cronk, 1977). The recommended nutrient intake of vitamin B12 (2.4 µg/day for general adult population (FAO/WHO, 2004)) would be met with moderate consumption of tempe.

Soy isoflavones are oestrogenic substances acting through human oestrogenic receptors, and as such they influence processes controlled by the female sex hormone estradiol. They have been associated with reduced prevalence of breast and prostate cancers, cardiovascular diseases and osteoporosis (Yuan, Wang, & Liu, 2007). Isoflavones, in total about 1.2–1.4 mg g⁻¹ (Góes-Favoni, Carrão-Panizzi, & Beleia, 2010), of which the glycosides daidzin, glycitin and genistin predominate in raw soybeans, can be decomposed into their corresponding aglycones daidzein, glycitein and genistein, by glucosidases of plant or microbial origin. The aglycones can be absorbed considerably better than their parent glycosides in the human digestive tract (Sepehr, Cooke, Robertson, & Gilani, 2009).

Research among 719 elderly (aged 68 and older) in Indonesia revealed that whereas tofu was associated with deteriorating memory (Hogervorst, Sadjimim, Yesufu, Kreager, & Rahardjo, 2008), the opposite occurred with consumption of tempe. Such different response was assumed to be caused by higher folate contents of tempe, although other bio-active components such as oestrogens (isoflavones) may have been involved (Hogervorst et al., 2008). However, controversial reports appeared in the literature either claiming the health effect of tempe or blaming the negative effects of consuming tofu (Hogervorst et al., 2008; Mangels, Messina, & Melina, 2003; White et al., 2000). The lack of knowledge of what precisely are the differences in nutrients and active ingredients between tempe and tofu, and the lack of standardization of the manufacturing processes as well as the use of different analytical methods across data published on tofu (Ginting et al., 2003; Wang & Murphy, 1996) and tempe (Arcot et al., 2002; Areekul et al., 1990; Ginting & Arcot, 2004; Keuth & Bisping, 1993) composition may all have played a role in the apparent confusion about the effectiveness of soy products (Albertazzi & Purdie, 2002). There is a scarcity of comparative studies on the nutritional difference between the two soy products, notwithstanding their consumption by billions of people.

The purpose of this investigation was to compare the contents and bioaccessibility of three types of bio-active components – folate, vitamin B12, and the isoflavone aglycones daidzein, glycitein and genistein – in tofu and tempe, each made under controlled

processing conditions from two commercially used soybean varieties.

2. Materials and methods

2.1. Materials

Soybeans: Yellow-seeded soybeans (*Glycine max*; Kleinjan, Rhoon, Netherlands) were used: Type I with a black hilum, used for commercial tempe making, and type II with a white hilum, used for commercial tofu making.

Micro-organisms: *Rhizopus microsporus* var. *microsporus* LU573 (Laboratory of Food Microbiology, Wageningen University) was grown on malt extract agar (MEA, Oxoid) at 30 °C for 1 week to obtain a well-sporulated culture. Sporangiospores were mildly rubbed into 9 mL peptone-physiological saline (PPS, peptone 1 g/L, NaCl 8.5 g/L) to obtain a suspension, of which 8 mL was used to inoculate 2 kg of cooked soybeans for tempe making. A commercial undefined powdered tempe starter [Raprima brand, Semarang, Indonesia] was also used for tempe making. This was stored at 22 °C, dry and in the dark. Inoculation was done according to manufacturer's instruction. *Lactobacillus plantarum* LU852 was grown and maintained in de Man, Rogosa and Sharpe (MRS, Oxoid) broth, with incubation at 30 °C for 48 h. It was used to inoculate soak water prior to adding raw soybeans for tempe making, at an inoculation level of 10⁶ CFU/mL of water.

3. Methods

3.1. Soybean processing

Four tempe making scenarios were designed to include variations in soaking and inoculation conditions, and a fifth scenario to represent tofu making, shown and specified in Fig. 1.

3.2. Sampling and sample treatment

All products were freeze-dried (GRInstruments, Wijk bij Duurstede, Netherlands), then ground (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany), sieved through a 0.5 mm sieve and stored at –20 °C.

3.3. Chemical analysis

Dry matter: Gravimetrically according to AACC method 44-15.02 (AACC, 2000); **Yield:** Calculated on a dry matter basis and expressed as % of raw material inputs; **pH:** Of liquids and solid samples alike, a 1:1 suspension was made with distilled water. The pH of the suspension was measured using a combination electrode (WTW, Weilheim, Germany); **Crude protein:** The DUMAS system was used according to the manufacturer's instruction (Flash EA 1112 protein analyzer, Thermo Scientific, Waltham, Mass.); **Amino Nitrogen:** According to Han, Kiers, and Nout (1999); **Crude fat and ash:** AOAC methods 27.006 14.006 (AOAC, 1984), respectively; **Folate:** Total folate was determined by microbiological assay using *Lactobacillus rhamnosus* ATCC 7469 as the growth indicator organism and 5-formyltetrahydrofolate as the calibrant (Kariluoto et al., 2004). The sample preparation procedure included heat extraction followed by trienzyme treatment with amylase, hog kidney conjugase, and protease (Kariluoto et al., 2004; Piironen, Edelmann, Kariluoto, & Bedö, 2008). Method performance was confirmed by analysing a certified reference material BCR 485 (Mixed vegetables; Institute for Reference Materials and Measurements, Geel, Belgium) in each set of samples. Action limits in the control charts were average $\pm 1.5 \times$ standard deviation. In addition, total folate

Fig. 1. Soybean processing scenarios for tempe and tofu. Legend: ¹I: Black hilum, for commercial tempe making, II: White hilum, for commercial tofu making; ²I dehulled by mechanical abrasion prior to soaking, II dehulled manually after soaking when used for tempe. ³Soaked natural: 24 h at 30 °C in tap water. ⁴Soaked acid: 24 h at 30 °C in tap water inoculated at start with 10⁶ CFU/mL of *Lactobacillus plantarum* LU852 pure culture. ⁵Soaked neutral: 16 h at 20 °C in tap water. ⁶Cooked in twice the weight of the soaked beans in fresh tap water for 20 min. ⁷Milled with tap water to a milky slurry (Condux werke, type LV15M, Wolfgang bei Hanau, Germany) at a dry bean:water ratio of 1:8; slurry was heated at 95–100 °C for 4 min, and filtered through a cheese cloth to obtain soymilk. ⁸Pure starter: Inoculated with 10⁶ CFU/g of *Rhizopus microsporus* var. *microsporus* LU573 pure culture. ⁹Mixed starter: Inoculated with 10⁶ CFU/g of commercial tempe starter [Raprima brand, Semarang, Indonesia]. ¹⁰Incubation at 30 °C. ¹¹Coagulation was at 70–80 °C by adding calcium sulphate (30 g/kg soybean dry matter) under agitation. The mixture was left for 10–15 min to complete the coagulation. The coagulate was pressed in cheese-cloth to remove excess soy whey. ¹²Cooked for 5 min in microwave oven without added water. ¹³Dynamic gastrointestinal system (TIM-1 system).

contents of duplicate samples were not allowed to differ more than 10%; **Vitamin B12:** Vitamin B12 was determined by a microbiological assay based on Kelleher and Broin (1991). The assay was performed using *Lactobacillus delbrueckii* ATCC 7830 as the growth indicator organism and cyanocobalamin as the external standard. A certified reference material BCR 487 (Pig's liver; Institute for Reference Materials and Measurements, Geel, Belgium) was analysed in each set of samples. As in folate analysis, action limits in the control charts were average $\pm 1.5 \times$ standard deviation. Vitamin B12 contents of duplicate samples were not allowed to differ more than 10%; **Isoflavones:** Purification according to Decroos, Vincken, van Koningsveld, Gruppen, and Verstraete (2007), followed by analysis and quantification according to Simons et al. (2011), using a gradient adapted as follows: 0–1 min, isocratic on 15% (v/v) B; 1–28 min, linear gradient from 15% to 60% (v/v) B; 28–29 min, linear gradient from 60% to 100% (v/v) B; 29–34 min isocratic on 100% (v/v) B; 34–35 min, linear gradient from 100% to 15% (v/v) B; 35–40 min isocratic on 15% (v/v) B; **In vitro bioaccessibility:** Products were digested in a multi-compartment dynamic computer-controlled gastrointestinal system (TIM-1 system, Fig. 2), simulating the stomach and small intestine described by Minekus, Marteau, Havenaar, and Huis in 't Velt (1995). Products were put into the stomach compartment with artificial saliva and water. Gastrointestinal secretions mimicking healthy adult after taking a meal (fed state) were described earlier (Mitea et al., 2008). During passage through the jejunum and ileum compartments, bioaccessible nutrients passed through the pre-filter and semi-permeable membrane, and the dialysates were collected every hour.

Fig. 2. Dynamic gastrointestinal system (TIM-1) Legend: 1. gastric intake; 2. jejunum dialysate; 3. ileum dialysate; 4. ileum efflux; 5a + 5b. stomach plus duodenum residue sample; 5c + 5d. jejunum plus ileum residue sample.

The non-bioaccessible compounds were collected as ileum effluents that would enter the colon. The residues in the system after a 6-h experiment under fed-state conditions were collected from the stomach, duodenum, jejunum and ileum. All products were

Table 1

Macronutrient composition of soybean products obtained through five processing scenarios.

	Dry matter (g/kg)		pH		Protein (g/kg DM)		Amino nitrogen ^b (mmol/kg DM)		Lipid (g/kg DM)		Ash (g/kg DM)	
Soybean type (I or II) ^a	I	II	I	II	I	II	I	II	I	II	I	II
Raw soybeans	931.3	927.8	6.56	6.52	402.1	386.0	2.6	2.6	231.5	186.4	48.0	49.8
<i>Scenario 1</i>												
Soaked natural ^c	397.1	361.2	4.90	5.12	425.5	437.1	2.0	3.2	284.4	214.2	27.2	39.1
Cooked natural	398.2	396.1	5.18	5.36	439.2	465.6	2.7	2.8	312.0	262.4	19.9	21.4
Tempe natural pure fermented 24 h ^d	423.8	392.9	4.99	5.46	454.8	481.8	3.2	3.4	305.2	266.3	19.9	22.3
Tempe natural pure fermented 48 h	391.1	359.8	6.33	5.63	459.7	462.7	5.8	6.0	285.1	231.6	19.6	21.8
Cooked tempe natural pure	481.1	580.5	6.35	6.35	462.9	495.2	5.7	5.3	281.7	245.3	20.9	22.5
<i>Scenario 2</i>												
Soaked natural	397.1	361.2	4.90	5.12	425.5	437.1	2.0	3.2	284.4	214.2	27.2	39.1
Cooked natural	398.2	396.1	5.18	5.36	439.2	465.6	2.7	2.8	312.0	262.4	19.9	21.4
Tempe natural mixed fermented 24 h ^e	386.7	401.0	5.10	5.30	448.5	483.6	3.4	3.8	301.0	257.6	19.6	20.8
Tempe natural mixed fermented 48 h	405.4	383.6	5.95	5.93	453.3	496.4	5.7	5.9	280.2	230.6	19.9	21.8
Cooked tempe natural mixed	583.3	526.3	5.77	6.21	453.0	483.0	5.1	4.8	289.3	245.3	21.3	21.8
<i>Scenario 3</i>												
Soaked acid ^f	357.9	356.8	4.18	4.75	419.3	440.5	2.9	2.8	266.9	248.2	30.9	38.5
Cooked acid	381.1	415.9	4.24	4.82	441.9	469.5	4.4	3.6	312.9	267.9	19.0	20.1
Tempe acid pure fermented 24 h	410.9	385.5	4.56	5.29	437.5	465.5	3.1	3.5	288.9	261.9	18.1	19.5
Tempe acid pure fermented 48 h ^g	379.1	399.5	6.08	5.80	449.5	489.3	6.8	5.8	277.3	233.5	19.8	21.1
Cooked tempe acid pure	488.0	616.2	6.19	6.23	441.7	494.3	5.4	5.1	291.9	243.3	19.2	20.9
<i>Scenario 4</i>												
Soaked acid	357.9	356.8	4.18	4.75	419.3	440.5	2.9	2.8	266.9	248.2	30.9	38.5
Cooked acid	381.1	415.9	4.24	4.82	441.9	469.5	4.4	3.6	312.9	267.9	19.0	20.1
Tempe acid mixed fermented 24 h	390.5	390.9	4.57	5.80	430.5	445.8	3.2	3.5	286.9	264.8	19.1	19.7
Tempe acid mixed fermented 48 h	347.9	382.7	5.77	5.21	443.3	489.6	6.0	6.0	275.9	242.2	19.4	20.9
Cooked tempe acid mixed	583.2	660.1	5.96	5.95	446.2	484.7	5.5	4.6	291.7	244.1	18.9	21.4
<i>Scenario 5</i>												
Soaked neutral for tofu ^h	382.9	362.6	6.60	6.62	414.5	395.0	2.4	2.8	258.9	200.6	42.2	40.6
Cooked tofu milk	73.4	68.9	6.60	6.59	465.4	390.2	3.6	3.1	187.9	217.8	62.7	45.0
Tofu whey	35.1	39.4	6.50	5.77	175.7	163.5	2.3	3.3	56.1	70.8	179.3	156.4
Tofu okara	206.5	182.8	7.13	6.98	307.0	314.9	2.3	2.4	225.3	174.5	37.5	39.0
Tofu ⁱ	168.8	173.2	5.98	5.87	472.9	509.2	3.1	3.3	237.4	213.7	77.8	105.0
Cooked tofu	268.0	269.2	6.05	5.83	503.3	517.6	3.2	3.1	263.7	220.4	78.2	103.3

^a I: Black hilum, used by tempe makers, dehulled by mechanical abrasion prior to soaking; II: White hilum, used for tofu making, dehulled manually after soaking when used for tempe.

^b mmol amino nitrogen/kg DM.

^c Soaked natural: 24 h at 30 °C in tap water.

^d Pure: *Rhizopus microsporus* var. *microsporus* LU573 pure culture.

^e Mixed: Commercial tempe starter [Raprima brand, Semarang, Indonesia].

^f Soaked acid: 24 h at 30 °C in tap water inoculated at start with 10⁶ CFU/mL of *Lactobacillus plantarum* LU852 pure culture.

^g Product yield 719 g/kg raw soybeans I (dry matter basis).

^h Soaked neutral for tofu: 16 h at 20 °C in tap water.

ⁱ Product yield 427 g/kg raw soybeans I (dry matter basis). All samples were analysed in duplicate, with duplicate measurements. Differences between duplicates did not exceed 10% of averages.

freeze-dried (GRInstruments, Wijk bij Duurstede, Netherlands), then ground (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany), sieved through a 0.5 mm sieve and stored at −20 °C.

3.4. Microbiological analysis

Enumeration of total viable microorganisms, lactic acid bacteria, Enterobacteriaceae, and fungi (yeasts and moulds) was done according to Zheng et al. (2012), and numbers were expressed as Log CFU/g sample. Microbial diversity was estimated by culture-independent direct DNA extraction using the FastDNA[®] Spin Kit for Soil (MP Biomedical, Solon, OH, USA), according to the manufacturer's protocol. 18S rDNA was amplified using primers NS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). A DNA clone library was made and sequencing (with forward primer NS1 (5'-GTAGT-CATATGCTTGTCTC-3')) was performed, both according to Lima et al. (2012).

All samples were analysed in duplicate, with duplicate measurements. Differences between duplicates did not exceed 10% of averages.

4. Results and discussion

The effect of processing on macronutrient composition is presented in Table 1. Scenarios 1–4 combined variations of soaking with or without added lactic acid bacteria, and fermentations using a pure fungal strain or a traditional fungal strain. Dry matter contents of tempe were consistently higher than in tofu; whereas absolute values for bean types I and II differed, the trend in dry matter contents as influenced by processing, was similar. This was also reflected in the yield based on dry matter of tempe (72%) which was higher than that of tofu (43%). Although a natural soak of soybeans in water resulted in a pH decrease, added lactic acid bacteria provoked a considerable acidification of soak water and beans, as was envisaged. During the stage of fungal fermentation, a gradual pH increase occurred, especially using the pure culture *Rhizopus* starter. On the other hand, tofu pH remained approximately neutral. During all five process scenarios a gradual increase of crude protein concentration occurred in the dry matter. Bean type II had a slightly lower crude protein content although this was not reflected in the final cooked products. The protein content levels for fresh tempe and tofu are of the same order as stated in the Netherlands food composition table (de Jong, 2012).

Table 2

Bacteriological characteristics of tempe obtained from beans type I through four processing scenarios.

(Log CFU/g)	Tempe scenario 1 ^a	Tempe scenario 2	Tempe scenario 3	Tempe scenario 4
Total viable count	8.9	9.0	6.7	7.1
Lactic acid bacteria	8.9	9.2	7.1	7.3
Enterobacteriaceae	4.4	2.8	<1.0	<1.0

^a Scenario 1: Natural soak with pure culture; scenario 2: Natural soak with mixed culture; scenario 3: Acid soak with pure culture; scenario 4: Acid soak with mixed culture.

Table 3Folate, vitamin B12 and isoflavone contents and *in vitro* bioaccessibility of soybean products obtained through five processing scenarios.

	Folate (µg/kg dry matter)		Vitamin B12 (µg/kg DM)		Daidzein (mg/kg DM)		Glycitein (mg/kg DM)		Genistein (mg/kg DM)	
	I	II	I	II	I	II	I	II	I	II
Soybean type (I or II)										
Raw soybeans	2671	2207	–	–	51	56	3	4	69	47
<i>Scenario 1</i>										
Soaked natural	1098	1604	–	–	263	406	15	76	390	397
Cooked natural	957	774	–	–	265	562	18	106	267	678
Tempe natural pure fermented 24 h	2264	2586	0.62	0.40	–	–	–	–	–	–
Tempe natural pure fermented 48 h	3604	4467	0.46	0.51	352	684	17	111	150	863
Cooked tempe natural pure	3484	4143	0.16	0.46	636	1391	28	223	1138	1388
<i>Scenario 2</i>										
Soaked natural	1098	1604	–	–	263	406	15	76	390	397
Cooked natural	957	774	–	–	265	562	18	106	267	678
Tempe natural mixed fermented 24 h	1826	2246	0.58	0.71	–	–	–	–	–	–
Tempe natural mixed fermented 48 h	1371	3079	0.83	0.72	442	1235	22	170	657	1170
Cooked tempe natural mixed	1493	2470	0.55	0.62	667	1100	40	214	1108	1507
<i>Scenario 3</i>										
Soaked acid	1915	1916	–	–	274	327	15	59	382	242
Cooked acid	1340	837	–	–	266	508	15	80	414	379
Tempe acid pure fermented 24 h	1889	2648	0.86	0.48	–	–	–	–	–	–
Tempe acid pure fermented 48 h	3904	5596	0.85	0.46	594	1370	28	183	830	1441
Cooked tempe acid pure	3153	3853	0.34	0.52	756	1271	35	197	1022	1492
Bioaccessible in cooked tempe acid pure	–	–	–	–	755	–	35	–	1021	–
<i>Scenario 4</i>										
Soaked acid	1915	1916	–	–	274	327	15	59	382	242
Cooked acid	1340	837	–	–	266	508	15	80	414	379
Tempe acid mixed fermented 24 h	1577	2001	0.63	0.85	–	–	–	–	–	–
Tempe acid mixed fermented 48 h	1625	3091	1.06	0.82	622	1310	30	240	1018	1676
Cooked tempe acid mixed	1619	2922	0.72	0.36	734	1281	39	199	1527	1608
<i>Scenario 5</i>										
Soaked neutral for tofu	1810	1604	–	–	–	–	–	–	–	–
Tofu okara	965	1184	–	–	–	–	–	–	–	–
Tofu	1254	797	–	–	608	1331	28	180	927	1407
Cooked tofu	1273	968	–	–	679	1265	29	150	959	1367
Bioaccessible in cooked tofu	–	–	–	–	679	–	29	–	959	–

Abbreviations as in Table 1: –, not determined. All samples were analysed in duplicate, with duplicate measurements. Differences between duplicates did not exceed 10% of averages.

The amino nitrogen concentrations showed a considerable increase between 24 and 48 h incubation during the tempe processes, indicating the degradation of peptide bonds by microbial proteases; without large differences between the starters used. In tofu, only a slight increase of amino nitrogen took place, as expected in a cooked product in which no enzyme activities would remain. Crude lipid concentrations did not change during the processes. During tempe processing a gradual lowering of the ash content occurred, in contrast with tofu in which ash increased considerably due to added coagulant.

In Table 2, bacteriological characteristics of the tempe scenarios 1–4 are summarised to demonstrate their possible relation with the formation of folate and vitamin B12. We observed that the objectives of the chosen scenarios were fulfilled, namely that the natural soak resulted in abundant growth of lactic acid bacteria but the relatively higher pH also allowed the growth of other bacteria such as Enterobacteriaceae; on the other hand, a strong

impact of *Lactobacillus plantarum* acidified soaking, with scenarios 3 and 4 having considerably less total viable bacteria, was reflected in similarly lower levels of lactic acid bacteria and absence of Enterobacteriaceae, the latter being in favour of food safety. Predominating lactic acid bacteria were *Enterococcus faecium*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Leuconostoc fallax* in decreasing order of abundance of sequenced DNA fragments; the latter was detected only in scenario 1. The undefined traditional starter contained *R. microsporus* var. *microsporus* (similar as our pure culture) and *R. microsporus* var. *chinensis*, with minor contaminations from soil fungi (*Cladosporium cladosporioides*) and Enterobacteriaceae, comparable to that of traditional starter “usar” used in Indonesia (Nout et al., 1992). In tempe scenario 1, the Enterobacteriaceae had been able to proliferate, but to low levels which may have been too low to have a significant impact on concentrations of metabolites such as vitamin B12.

Fig. 3. Folate *in vitro* dynamics of bioaccessibility.

Table 3 shows folate, vitamin B12 and isoflavone aglycone concentrations during soybean processing. Raw soybeans contained a considerable amount of folate, 2671 and 2207 $\mu\text{g/kg}$ DM in beans type I and II, respectively. Folate values have been reported, ranging from 1380 to 4500 $\mu\text{g/kg}$ DM (Arcot et al., 2002; Ginting & Arcot, 2004; Ginting et al., 2003), depending on variety, growing and storage conditions, and method of folate analysis. Folate loss by soaking was higher in natural (neutral) soaking (scenarios 1, 2, and 5: 27–59%) than in the acidified soaking (scenarios 3 and 4: 13% and 28%, respectively) and also higher for beans I than for beans II. Average loss in soaking, 32%, was comparable to previous studies reporting 19–31% losses (Arcot et al., 2002; Ginting et al., 2003). In tempe preparation, cooking after soaking caused further folate losses to only 35–50% of initial folate contents of the native soybeans. Retention after neutral soaking and cooking (35–36%) was similar to 37% retention reported by Arcot et al. (2002) although the soybeans they used had significantly higher folate content.

During tempe fermentation folate increased, compensating for the losses and balancing out the differences caused by different soaking treatments. Scenario 3 (acid pure) gave the largest increase (factor 2.9 and 6.7 for beans I and II, respectively). Similar increase during fermentation (four to fivefold) was reported by Murata, Miyamoto, Kokufu, and Sanke (1970). The lowest tempe folate levels were found in scenario 2 (natural mixed), with 1.4-fold (I) and 3.9-fold (II) increases. Microwave cooking for 5 min decreased folate content by 0–31%, which is of same magnitude as 21% loss in deep-frying reported by Arcot et al. (2002). Comparative folate levels in cooked products showed that with scenario 3 indexed as 100%, scenario 2 had 47% (I) and 64% (II), and scenario 5 (tofu) 40% (I) and 25% (II). Foliates are stable at the pH range 4–8 (De Brouwer, Zhang, Storozhenko, Van der Straeten, & Lambert, 2007) but *Lactobacillus plantarum* was shown to synthesize folates (Rossi, Amaretti, & Raimondi, 2011), which suggests that folate levels in tempe are strongly determined by microbial activities. In addition to the starter, the variety seemed to have an effect: Tempe prepared from beans II had always higher folate contents than those where beans I had been used. Thus, the original folate content of soybean did not correlate with the folate content of the final products. Possible explanations include the difference in bean structure (for instance, the proportion of hulls and cotyledons and folate distribution between them) or perhaps different carbohydrate profiles affecting the fermentability by the mould, and hence, the folate synthesis capacity.

The retention of folate in tofu (compared to unprocessed soybeans) was only 36–47%. The retention as well as the final folate contents (797 $\mu\text{g/kg}$ DM and 1254 $\mu\text{g/kg}$ DM) were in good agreement with Ginting et al. (2003) who reported 40% retention and a folate content of 1084 ± 117 $\mu\text{g/kg}$ DM. Retention may be regarded

small, but nevertheless, tofu is a good source of folate and makes a considerable contribution to folate intake in Asian and vegan diets. In addition, okara had similar high folate content as tofu, which encourages its use as a nutritious ingredient in other food applications.

Since tempe scenarios 1 and 3 resulted in higher folate contents than in 2 and 4, we selected one of the former (scenario 3) for our limited trials in the TIM-1 system, to compare with scenario 5 (tofu). Fig. 3 represents the bioaccessibility of folate in the TIM-1 *in vitro* digestion simulation. Recovery rates in the TIM system were calculated by setting folate output (folate in dialysates, efflux, and residues) against folate input (folate in test product and endogenous folate deriving from the TIM secretion). Recoveries were 97% for tempe and 103% for tofu, demonstrating good performance of the system. Tempe combines a higher folate content with a better bioaccessibility, compared with tofu. However, the 82% bioaccessibility from tofu can still be regarded high and is comparable for instance to folate bioaccessibility from yoghurt fortified with folic acid or 5-methyltetrahydrofolate (Arkbåge, Verwei, Havenaar, & Witthöft, 2003). We did not further investigate the reasons for the slightly lower folate bioaccessibility in tofu; that may have resulted from differences in the food matrix of tempe vs. tofu.

Vitamin B12 was determined only in fermented products. Tempe scenarios 1–4 gave rise to similar vitamin B12 biosynthesis. Although nutritionally significant amounts of B12 have been reported for tempe (Areekul et al., 1990; Keuth & Bisping, 1993; Liem et al., 1977), the low B12 content in tempe in our study was not surprising. There are several publications stating that *Rhizopus* does not produce vitamin B12; instead, bacteria associated with tempe starters can be responsible for the vitamin B12 synthesis. Areekul et al. (1990) found that accompanying bacteria such as *Klebsiella pneumoniae* could produce vitamin B12, and Keuth and Bisping (1993) reported that addition of *K. pneumoniae* and *Citrobacter freundii* resulted in an increase of vitamin B12 concentration in tempe. We noticed low numbers of Enterobacteriaceae in scenarios 1 and 2, but none in the other products, irrespective of the type of fermentation starter used. The type of beans used caused only slight differences among the products. This indicated that the formation of vitamin B12 was not critically determined, neither by the chosen scenarios nor by the type of soybeans. After cooking, 32% of the vitamin B12 was lost.

The predominant soybean isoflavone aglycones and their level of bioaccessibility are presented in Table 3. We observed a strong effect of the bean type. This effect of variety was reported elsewhere previously (Wang & Murphy, 1996). The strong increase of aglycones during the soaking of soybeans suggests that β -glucosidases were activated during soaking and were responsible for the degradation of the parent glycosides daidzin, glycitin and genistin. It is not clear whether these enzyme activities were endogenous in soybeans, or produced by e.g. lactic acid bacteria (Hubert, Berger, Nepveu, Paul, & Dayde, 2008); it was suggested that the water absorption capacity of soybean varieties affects the leaching of isoflavones in water, as well as the activation of glucosidases (Toda, Sakamoto, Takayanagi, & Yokotsuka, 2001). An even larger increase of aglycone concentrations was observed during the tempe fermentation, indicating the effect of microbial β -glucosidase activity since the cooked soybeans must have lost all of their endogenous enzyme activity. Differences between pure and mixed starter were only very small and may be the result of analytical variation. In tofu (scenario 5) comparable levels of aglycones were observed which indicated that also without fermentation, promising levels of isoflavone aglycones could result from processing. We hypothesize that a relatively more effective degradation of glycosides in soaked beans occurred during the grinding which is required to extract soymilk for tofu making. The particle size reduction of

soybeans would greatly enhance diffusion of glycosides and glucosidases and thus would result in more effective aglycone formation than in intact soybean cotyledons. We observed a high bioaccessibility of the aglycones present in tempe as well as in tofu. The fact that aglycones were better absorbable than glucosides has been stated (Izumi, Osawa, Obata, Tobe, & Saito, 2000), and it was reported that about 75% of isoflavones of soybean foods were absorbed *in vivo* (Xu, Wang, Murphy, & Hendrich, 2000).

5. Concluding remarks

In conclusion, the effect of processing, particularly fermentation, was reflected in folate and vitamin B12 contents. The effect of bean variety was mainly demonstrated in the isoflavone aglycone content of processed soybeans. Although tofu has lower contents of folate and vitamin B12 than tempe, it nevertheless makes an important contribution to folate intake in the Asian dietary context.

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