# Effects of tempeh fermentation using *Rhizopus oryzae* on the nutritional and flour technological properties of *Zamnè* (*Senegalia macrostachya* seeds): Exploration of processing alternatives for a hard-to-cook but promising wild legume


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**Abstract**

*Zamnè* is a promising wild, healthy, but hard-to-cook legume in the drought- and hunger-prone areas of West to Northcentral Africa. The aim of this study was to explore processing alternatives for *Zamnè* and mitigate its hard-to-cook defects (*i.e.*, the lixiviation of most soluble, bioaccessible, or digestible nutrients after the compelled extensive cooking). Therefore, *Zamnè* was fermented into tempeh using *Rhizopus oryzae*, and the effects of the fermentation on its nutritional values, digestibility, and flour technological properties were assessed. The fermentation for 48 h (*i.e.*, fresh tempeh) caused significant decreases in lipid (by 30%) and insoluble dietary fiber (by 22%) contents and antioxidant activity (by 24%). Interestingly, it resulted in a complete elimination of phytate and significant increases in the degree of protein hydrolysis (by 155%), zinc bioaccessibility (by 173%), soluble dietary fibers (by 315%), and soluble phenolics (by 46%). The overripening of the product for 72 h caused only a further decrease in the lipid content (by 26%) and a significant reduction of the protein hydrolysis degree (by 31%). Furthermore, the fermentation considerably altered the color (from yellow to brown) and significantly improved the yield (92%–94%), the water absorption index (4.3), the water solubility index (23%–24%), and the emulsion activity (53%-60%) of the flour. It could be concluded that tempeh fermentation can mitigate the hard-to-cook defects, improve the nutritional values, digestibility, and key flour technological properties of *Zamnè*, upgrade its usability, and facilitate its promotion in human diets.

**Keywords**: Flour, Hard-to-cook problem, Legume, *Rhizopus oryzae*, *Senegalia macrostachya*, Solid-state fermentation, Tempeh, *Zamnè*

## 1. Introduction

*Zamnè* (*Senegalia macrostachya* (Reichenb ex DC) Kyal. & Boatwr seeds) is a promising wild but underutilized legume in the drought- and hunger-prone areas of West to Northcentral Africa.

*Zamnè* has been identified as an important source of protein (20-30 g/100 g dry matter), dietary fibers (16-30 g/100 g dry matter), and bioactive non-starch polysaccharides and cryptic peptides (Drabo et al., 2020; Patent No. EP 2 506 723 B1, 2017; Zongo et al., 2022). It is used in Burkina Faso as a traditional food and delicacy and is receiving attention for developing functional foods and nutraceuticals (*e.g.*, for individuals with diabetes, gastrointestinal diseases, and cardiovascular diseases) (Ganaba, 2017; Patent No. EP 2 506 723 B1, 2017; Zongo et al., 2022). However, *Zamnè* exhibits a hard-to-cook problem and implies long cooking time (3–5 h), high energy expenditure, substantial loss of nutrients (associated with the extensive cooking), low protein, carbohydrate, and mineral digestibility, and low adoption in human diets (Drabo et al., 2020, 2023; Hama-Ba et al., 2017). In fact, the hard-to-cook problem of legumes is associated with their hardness or resistance to cooking and hard-to-disrupt or -digest cell wall structures, making their processing in general difficult and compromising their nutritional properties or digestibility (Gwala et al., 2020; Mubaiwa et al., 2017; Reyes-Moreno et al., 1993). Given the hard-to-cook defects (*i.e.*, the lixiviation of most soluble, bioaccessible, or digestible nutrients after the compelled extensive cooking), processing alternatives are needed to diversify, facilitate, and promote the use of *Zamnè*.

Fortunately, several processing alternatives, including pre-soaking techniques, roasting, milling, pressure cooking, extrusion cooking, germination, and solid-state fermentation, have been developed and demonstrated to improve the nutritional properties and facilitate the use of hard-to-cook legumes (Mubaiwa et al., 2017; Reyes-Moreno et al., 1993). Amongst all those processing alternatives, tempeh fermentation (a solid-state fermentation steered up by *Rhizopus* spp.), originated from Indonesia, is trending worldwide as a low-cost, health-promoting, and sustainable food processing technology to develop protein-rich foods from legumes and food by-products (Ahnan-Winarno et al., 2021; Nout & Kiers, 2005). It has been demonstrated that during tempeh fermentation, the mold (*i.e.*, *Rhizopus spp.*) synthesizes essential nutrients and

bioactive compounds (*e.g.*, vitamins, non-proteinogenic amino acids), produces an array of enzymes (*e.g.*, carbohydrases, protease, lipase, and phytase) that pre-digest the substrate, degrade major antinutritional factors (*e.g.*, flatulence sugars, phytate, and allergens), and improves the solubility and bioaccessibility of nutrients (Ahnan-Winarno et al., 2021; Nout & Kiers, 2005). Therefore, tempeh fermentation could facilitate the disruption of the strongly tied cell walls in hard-to-cook legumes, such as *Zamnè*, and improve the bioaccessibility and digestibility of the nutrients (Nopharatana et al., 2003; Reyes-Moreno et al., 1993). Moreover, tempeh is receiving increasing attention as a promising alternative to meat and a versatile product for healthy food formulations for all ages, including toddlers' food supplements (*i.e.*, porridge flours and beverages, contrarily to the traditionally cooked *Zamnè*) (Ahnan-Winarno et al., 2021).

On the other hand, milling also receives considerable attention as a key process for the development of convenient food products from legumes (Du et al., 2014; Garrido-Galand et al., 2021; Reyes-Moreno et al., 1993). However, though legumes, such as *Zamnè*, flours are potential sources of high-quality dietary protein and multifunctional and healthy food ingredients, they had been disregarded for a long time due to principally their antinutritional factors (*e.g.*, phytate, tannins, and trypsin inhibitors), poor technological or functional properties (*e.g.*, water holding capacity, bulk density, and gelation ability), and unappealing flavors (Du et al., 2014; Garrido-Galand et al., 2021). Different pre-treatments, including soaking, precooking, and fermentation, have been explored to improve the milling properties (*e.g.*, flour yield), reduce the antinutritional factors, and enhance the nutritional, sensory, and technological properties of legumes. Suffice it to say, fermentation, including tempeh fermentation, stood out again as one of the most promising pre-processing options before milling (Ahnan-Winarno et al., 2021; Garrido-Galand et al., 2021). The other way around, food formulations from tempeh flour have shown better acceptance than the original mold-knitted

cake in areas where people are not familiar with tempeh yet, including Burkina Faso, where *Zamnè* is currently consumed (Ahnan-Winarno et al., 2021). The versatile usability of tempeh flour makes the combination of those two processes a promising and innovative processing alternative for legumes, particularly the hard-to-cook ones such as *Zamnè*.

Alternative, health-promoting, sustainable, and affordable foods, such as wild plant-based foods, *Zamnè*, and tempeh, are resolutely researched to tackle food and nutrition insecurity worldwide, considering particularly the exacerbating crises (*i.e.*, climate disasters, epidemics, economic downturns, social conflicts, and food insecurity) in the last decades (Ahnan-Winarno et al., 2021; FAO/IFAD/UNICEF/WFP/WHO, 2022; Mariutti et al., 2021; Willett et al., 2019). However, notwithstanding its food potential, *Zamnè* remains primarily merely boiled for consumption. Only Guissou et al. (2020) explored alternative processing of *Zamnè* and fermented it into a *Soumbala*-like product. Soumbala is originally a West African traditional condiment or taste enhancer (*i.e.*, glutamate flavor) made from alkaline fermentation (*i.e.*, steered by *Bacillus* spp.) of *Parkia biglobosa* seed kernels. In line with the endeavor of developing alternative, health-promoting, sustainable, and affordable food products, this study aimed to explore *Zamnè* fermentation into tempeh using *Rhizopus oryzae*, mitigate its hard-to-cook defects, and assess the effects of the fermentation on its nutritional values, digestibility, and flour technological properties.

## 2. Material and methods

### 2.1. Seed sample and starter culture

*Senegalia macrostachya* (Rchb. ex DC.) Kyal. & Boatwr. mature and dry seeds (5 kg) were harvested from the wild (N 13.09 W 03.12, Burkina Faso) in December 2018, and a specimen was identified and deposited at the herbarium (INFOBIO N° 6887) of University Joseph Ki Zerbo (Ouagadougou, Burkina Faso). The seeds were immediately shipped to VEG-i-TEC (Ghent University, Kortrijk Campus, Belgium) in airtight plastic bags and stored at -20°C until

analysis. *Rhizopus oryzae* starter powder (grown on rice and soy-free) was purchased from Culture for Health (San Francisco, California, USA).

### 2.2. Reagents

Vinegar (8% acidity) and peanut oil were purchased from a local market in Kortrijk, Belgium. Sabouraud dextrose (SDA) and total plate count (PCA) agars, bacteriological peptone, and chloramphenicol supplement were purchased from Oxoid (Merelbeke, Belgium). Ferric chloride, gallic acid, phytic acid (P-8810), trolox, 2,2′-bipyridine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin and Ciocalteu′s phenol, thioglycolic acid, α-chymotrypsin from bovine pancreas (type II, ≥40 U/mg), trypsin from porcine pancreas (type IX-S, 13,000-20,000 U/mg), pancreatin from porcine pancreas (8xUSP), pepsin from porcine gastric mucosa (3200–4500 U/mg), porcine bile extract, protease from *Streptomyces griseus* (type XIV, ≥3.5 U/mg), and protease from *Bacillus licheniformis* (type III, 7.5–15 U/mg) were purchased from Sigma-Aldrich (St Louis, MO, USA). L-Leucine and 2,4,6-trinitrobenzene sulfonic acid (5% w/v) were purchased from Acros Organics (Leicestershire, England) and ThermoFisher Scientific (Waltham, MA, USA), respectively. Available carbohydrates (K-AVCHO 01/2021) and ammonia (Cat. No. 11 112 732 035) assay kits were purchased from Megazyme (Dublin, Ireland) and R-Biopharm (Darmstadt, Hess, Germany). All the reagents were of analytical grade.

### 2.3. Tempeh production

The seeds were precooked and fermented following the simplified tempeh fermentation process outlined in **Fig. 1**. Briefly, 500 g seeds were weighed, transferred into a stainless steel pot containing pre-boiling distilled water (at a seeds-to-water ratio of 1/3 (w/v)), and boiled (98 ± 2°C) for 90 min. Then, the parboiled seeds were drained, washed using lukewarm distilled water to eliminate the thick mucilage, and boiled (98 ± 2°C) again for another 90 min, as described earlier. The seeds-to-water ratio was monitored and maintained during the cooking process

using lukewarm distilled water. After the second boiling, the seeds were drained, spread out in a hot and open stainless steel pan, and stirred until complete elimination (~10 min) of the superficial moisture. Subsequently, the seeds were cooled down to approximately 40–50°C, mixed with vinegar (8% acidity) and the starter powder (*R. oryzae*) (1.5 mL vinegar/150 g seeds and $10^{4-5}$ viable spores/g seeds), and packed as a 1-inch bed in zip-lock plastic bags with pin holes spaced of 1 inch. Different bags were prepared and incubated, each presenting a time point for analysis. The incubation was done between 0 and 120 h at 30°C and 70% relative humidity using a PharmaEvent stability test chamber (Weisstechnik, Liedekerke, Belgium).

### 2.4. Assessment of the produced tempeh quality

According to Ahnan-Winarno et al. (2021), the key determinants of the fermentation process (*i.e.*, mold growth and biomass yield, moisture, $a_w$, pH, and ammonia production) and the tempeh quality (*i.e.*, microbial quality, seed coverage by the mold, and nutritional properties) were immediately assessed when the products were withdrawn from the climate chamber at different time points (0, 30, 36, 42, 48, 72, 96, 120 h) as follows.

#### 2.4.1. Determination of the mold biomass yields

The weights of the packs were measured at 0 h before the incubation (M0) and at the time of withdrawal (Mt) from the climate chamber (meaning after fermentation), and the mold biomass yields were calculated based on the mass balance: Biomass yield (%) = 100 x (Mt (g) x dmt)/(M0 (g) x dm0) - 100, where dm0 and dmt are the dry matter contents (%) of the products at 0 h and after the fermentation.

#### 2.4.2. Assessment of the mold growth and the microbial quality

Mold and total mesophilic microflora were assessed by plating on SDA-chloramphenicol and PCA agars following ISO 21527–1 and ISO 4833-2, respectively. Accordingly, immediately after the withdrawal from the climate chamber, 10 g of the fermented seeds were weighed in sterile stomacher bags and homogenized for 1.5 min with 90 mL of sterile saline peptone buffer

(8.5 g NaCl and 1 g of bacteriological peptone in 1 L of distilled water) using an IUL 0470 masticator (Led techno, Heusden-Zolder, Belgium). Then, 100 µL of the initial suspension was streaked on duplicates of PCA. Besides, adequate decimal dilutions (order 2-5) were streaked on a duplicate of SDA-chloramphenicol plates. Afterward, the PCA plates were incubated under aerobic conditions at 30°C for 3 days, and the SDA plates were incubated under aerobic conditions but at 25°C for 24 h. The SDA plates were checked and counted after 24 h instead of 5 days, as recommended by ISO 21527–1, since the mold was overgrowing after 24 h.

### 2.4.3. Assessment of the mold coverage of the products

In compliance with the International Commission on Illumination (ISO/CIE 11664-4:2019), the CIELAB color coordinates (L*, a*, and b*) of the products were measured using the spectrophotometer ColorFlex EZ 0130 (Hunterlab, Reston, VA, USA), providing an estimate of the seed coverage by the mold. The apparatus was set at illuminant D65, standard observer 10°, and 45°/0° geometry (*i.e.*, illumination/viewing angles), and then calibrated using black glass and white tile. The surface sides of triplicate slices (4 cubic inches each) of the fermented products and 3 g of the unfermented seeds (*i.e.*, after inoculation but at time 0 h) were placed on the sample port and measured. The Chroma value (C) and the whiteness index of Judd (WI) were calculated according to Hirschler (2012):

$$C = \sqrt{a*^2 + b*^2} \qquad (\mathbf{eq.1})$$

$$WI = 100 - \sqrt{(100 - L*)^2 + a*^2 + b*^2} \qquad (\mathbf{eq.2})$$

### 2.4.4. Measurement of the water activity ($a_w$)

The water activity ($a_w$) was measured according to ISO 18787 and using AQUA LAB 4TE (Meter group, Munich, Bavaria, Germany). Briefly, the calibration of the apparatus was checked using ultrapure water ($a_w$ = 1) and 2.33 mmol/mL NaCl solution ($a_w$ = 0.920) at room temperature (25°C). After the verification, a slice of each product (2.5 cm x 2.5 cm x 1 cm) was

separately placed in the measurement chamber, and the water activity was read after the equilibration of the product and the surrounding atmosphere inside the measurement chamber was reached.

### 2.4.5. Determination of the pH and ammonia content

The precooked seeds and fermented products were finely mashed using a mortar and pestle, and then the pastes (1 g) were immediately suspended in 9 mL of distilled water, and the pHs were measured using pH-meter Hanna HI2002-02 (Hanna instruments, Temse, Belgium), according to Terlabie et al. (2006). The ammonia content was determined using the ammonia assay kit. Briefly, 10 g of the mashed sample was suspended in 20 mL of 1 M perchloric acid, vigorously vortexed for 2 min, and diluted with 40 mL of distilled water. Then, the suspensions were adjusted to pH 7.0–7.5 with 5 M KOH, and the volume was adjusted to 100 mL using distilled water. Subsequently, the suspensions were filtered (0.5 µm filter), and aliquots (0.1 mL) of the adequately diluted filtrates were transferred into cuvettes. Distilled water, instead of filtrated suspensions, was used as a blank. Then, 1 mL NADH (0.4 mg NADH/mL of triethanolamine buffer (pH 8) containing 2.5 mg/mL of 2-oxoglutarate) and 1.9 mL of distilled water were added. The mixtures were incubated for 5 min, and the absorbance was recorded at 340 nm (A1) using a spectrophotometer (Shimadzu UV 1800) (Shimadzu, Duisburg, North Rhine-Westphalia, Germany). Finally, 0.02 mL of glutamate dehydrogenase solution (1000 U/mL) was added, and then the mixtures were incubated for another 20 min. The absorbances were recorded again at 340 nm (A2), and the ammonia contents were calculated as follows:

$$\text{Ammonia (mg/100 g dry matter)} = \frac{100 \text{ x D x Vs x Vr x MW x } \left((\text{A2} - \text{A1})\text{sample} - (\text{A2} - \text{A1})\text{blank}\right)}{\varepsilon \text{ x d x Va x m x dm}} \qquad (\mathbf{eq.3}),$$

where D = dilution factor, Vs = volume of the extract (100 mL), Vr = volume of the reaction mixture (3.02 mL), Va = volume of the analyzed aliquot (0.1 mL), MW = molecular weight of

ammonia (17.03 g/mol), ε = extinction coefficient of NADH at 340 nm (6.3 $L \times mmol^{-1} \times cm^{-1}$), d = light path (1 cm), and m = weight of the sample (g), and dm = dry matter content (%). Afterward, the remaining seed pastes were stored at -20°C until further analysis.

### 2.4.6. Assessment of the nutritional properties

The moisture contents of the samples were assessed according to ISO 1442. After that, the nutritional composition of the precooked seeds (with the starter but sampled at time 0 h) and the developed fresh tempeh (fermented 48 h) and overripe tempeh (fermented 120 h) were analyzed. Total ash, lipid, and protein contents were determined following ISO 2171, 11085, and 937, respectively. The nitrogen-to-protein conversion factor (NPCF) of 4.05 was used as a weighted average of the specific NPCFs of traditionally cooked *Zamnè* (*i.e.*, 4.06) (Drabo, 2023) and *R. oryzae* biomass (*i.e.*, 4.03) (Ibarruri & Hernández, 2019) (*i.e.*, (82 x 4.06 + 18 x 4.03)/100, where 82 and 18 refer to the seed and mold fractions in the final tempeh product). Iron and zinc contents were measured using inductively coupled optical emission spectrometry (iCAP 7200 ICP-OES) (ThermoFisher Scientific, Langenselbold, Hess, Germany), as described by Gabaza et al. (2018). The available carbohydrates (A-CHO) (*i.e.*, glucose, fructose, and galactose) and insoluble dietary fibers (IDF) contents were determined according to AOAC 2020.07 (McCleary & McLoughlin, 2021) and 2017.16 (McCleary, 2019), respectively. The total carbohydrates (T-CHO), total dietary fibers (TDF), and soluble dietary fibers (SDF) contents were calculated as follows: T-CHO (g/100 g dry matter (dm)) = 100 – ash – lipid – protein, TDF (g/100 g dm) = T-CHO – A-CHO, and SDF (g/100 g dm) = TDF – IDF. Finally, the metabolizable energy contents were calculated using the extensive general factor system (WHO/FAO, 2003) as follows: metabolizable energy content (kcal/100 g dm) = 9 x lipid + 4 x protein + 3.75 x A-CHO + 2 x TDF.

Subsequently, the antioxidant activity, antinutritional factor (*i.e.*, phytate), and digestibility of the products were assessed. The soluble phenolic compounds were extracted in duplicate and

quantified as gallic acid equivalent using the Folin-Ciocalteu assay, as described by Phuong et al. (2020). Then, the antioxidant activities of the extracts were measured by performing the DPPH assay and expressed as trolox equivalent (Phuong et al., 2020). The phytate contents were determined by following the modified Haug and Lantzsch method (Reichwald & Hatzack, 2008). The digestion of the products was simulated according to INFOGEST 2.0 (Brodkorb et al., 2019), and the iron and zinc bioaccessibilities were determined, as described by Gabaza et al. (2018). The digestibility of the proteins was determined using the pH drop method (Hsu et al., 1977), and the degree of protein hydrolysis in the digests was determined by measuring the primary amine contents according to Adler-Nissen (1979). L-leucine was used as a primary amine standard.

### 2.5. Processing of flours and evaluation of their technological properties

The flours were processed and analyzed according to Kaur and Singh (2005). Samples (~50 g) of the precooked seeds (without starter) and the developed fresh and overripe tempeh products were mashed using a mortar and pestle, dried for 72 h using a freeze dryer (Labconco, Kansas, MO, USA), and ground using the mortar until no material could pass through a 215 µm sieve (VWR, Leuven, Belgium). The extracted flours and remaining brans are presented in **Fig. 2**. The flour yields were calculated by dividing the extracted flour weight (g) by the freeze-dried sample weight (g) x 100. The colors of the flour samples (5 g) were measured as described in **section 2.4.3**. Then, a graduated cylinder of 10 mL was weighed (Mi) and filled with the flour. The cylinder was gently tapped on the bench until there was no further diminution of the sample level after filling to the mark. The filled cylinder was finally weighed (Mf), and the bulk density was calculated as follows: Bulk density = (Mf (g) – Mi (g))/10 mL.

Afterward, samples (1.5 g) of the flours were weighed in a falcon, mixed with 18 mL of water, heated for 15 min at 90°C in a warm water bath, cooled to room temperature (25°C), and centrifuged at 3000 g for 10 min. Then, the supernatants were collected in a tared aluminum

foil dish and dried overnight at 105°C in a forced air oven. The sediments were immediately weighed, and the water absorption indices (WAI) and solubility indices (WSI) were calculated as follows: WAI (g/g) = sediment weight (g)/flour sample weight (g) and WSI (%) = 100 x weight of the solids in the supernatant (g)/flour sample weight (g).

Subsequently, samples (0.7 g) of the flours were suspended in 10 mL of distilled water and whipped at 13,000 rpm for 30 seconds using Ultraturrax IKA T18 (IKA, Staufen, Baden-Württemberg, Germany). Then, 5 mL of peanut oil was added, and the mixtures were whipped again at 13,000 rpm for 30 seconds. Another 5 mL of the oil was added, and the mixture was whipped for 90 seconds. Finally, the emulsions were centrifuged at 1100 g and 25°C for 5 min, and the emulsion activities (EA) were calculated by dividing the volume of the emulsion layer (mL) by the total mixture volume (mL) x 100. After that, the emulsions were heated for 15 min at 85ºC, cooled at room temperature (25°C), and centrifuged again at 1100 g and 25°C for 5 min, and the emulsion stability (ES) was calculated by dividing the volume of the emulsion layer after heating (mL) by the volume of the emulsion layer before heating (mL) x 100.

Last but not least, samples (0.45 g) of the flours were suspended in 15 mL of distilled water, whipped with Ultraturrax IKA T18 for 3 min at 20000 rpm, and transferred to a graduated cylinder of 25 mL. Then, the disperser tube was cleaned with 3 mL of water and collected in the cylinder. The foam volumes were recorded immediately and after 120 min (at room temperature (25°C)), and the foaming activities (FA) and stabilities (FS) were calculated as follows: FA (%) = 100 x foam volume at 0 min (mL)/volume of the suspension before whipping (mL) and FS (%) = 100 x foam volume after 120 min of standing (mL)/foam volume at 0 min (mL).

### 2.6. Data analysis

All the statistical analyses were realized using R program version 4.2.0 (R Core Team, 2022). The seeds were fermented on two separate occasions, and the data were subjected to a one-way

analysis of variance (ANOVA) and Tukey–Kramer multiple rank test, according to Granato et al. (2014). The significance of differences was accepted at p-value < 0.5.

## 3. Results and discussion

### 3.1. Fermentation process and characterization of the fresh and overripe tempehs

**Fig. 3** displays the changes in selected determinants during the fermentation of *Zamnè* into tempeh. During the fermentation process, the colony-forming units of the mold increased from 0 h ($10^5$ CFU/g) to 42 h ($10^8$ CFU/g) and then slightly decreased until 72 h ($10^7$ CFU/g), to further remain stable up to 120 h (**Fig. 3A**). The fermentation process was associated with substantial mold biomass production (9%-18% dry matter) after 36 h, and no further significant changes were observed afterward (**Fig. 3A**). Meanwhile, while the pH and the ammonia contents of the products only started to change after 30 h and steadily increased (from 5.6 to 7.2 and 97 to 1503 mg $NH_3$/100 g dry matter, respectively) until 120 h (**Fig. 3B**), the moisture content and the water activity ($a_w$) steadily decreased until 120 h (from 81% to 74% and 0.98 to 0.995, respectively) (**Fig. 3C**). Besides, as illustrated in **Fig. 4** and with the whiteness indices of the products (**Fig. 3D**), the mold steadily covered the seeds, providing well-compacted cakes after 36 h of fermentation. Then, the whiteness of the mold started to fade after 48 h of fermentation as determined by the chroma values (**Fig. 3D)**. Yet, the contrast between the chroma values (which do not account for the brightness (L*) of the products) (**Fig. 3D**) and the color projections (**Fig. 4**) could be explained by the uncovered seeds that were still visible before 48 h of fermentation.

As shown, the progression of the fermentation and changes in the selected determinants (*i.e.*, $a_w$, ammonia production, pH, mold growth and color change, seed coverage by the mold, and biomass yield) were all relatively in line with the fermentation of soybean into tempeh, tempeh fermentation in general, and particularly fermentation with *R. oryzae*, as reported before in previous studies (Ahnan-Winarno et al., 2021; Erkan et al., 2018), demonstrating an excellent

fermentability of *Zamnè* into tempeh. More interestingly, no aerobic and mesophilic bacteria could be detected in the different products (detection limits of $10^2$ CFU/g of sample, respectively), ensuring a good hygienic control of the fermentation process. Accordingly, an optimal or fresh *Zamnè* tempeh was identified after 48 h of fermentation, characterized by a profuse growth of the mold, complete coverage of the seeds with the whitest cottony mold, compact cake form, and pH increase to 6.3. Then, the product started aging or overripening gradually by producing more ammonia, increasing the pH (above 7.0) further, and turning grayish (*i.e.*, a sporulation phase of the mold).

In contrast, variable fresh and overripe tempeh fermentation times (24–48 h and 72–120 h, respectively) have been reported depending on the substrates used, the starter culture choice, and the fermentation conditions (*e.g.*, setting, temperature, relative humidity) (Ahnan-Winarno et al., 2021; Erkan et al., 2020; Polanowska et al., 2020). The standard for tempeh production (CODEX STAN 313R-2013) has not defined the durations and phases (*i.e.*, maturation, aging, and deterioration) of the fermentation yet and manifestly requires further specifications, particularly considering the diversity of the substrates, the starter cultures, the fermentation conditions, and the methods of analysis of the fermentation process and products. Nevertheless, fresh and overripe *Zamnè* tempehs will certainly have different sensory and nutritional properties, health benefits, and food uses (Ahnan-Winarno et al., 2021). In fact, overripe tempeh is mainly used as a seasoning ingredient due to its strong flavors (*e.g.*, pungent aroma and umami or glutamate taste) (Ahnan-Winarno et al., 2021; Gunawan-Puteri et al., 2015) – similar to *Soumbala,* originally a West African traditional condiment made from alkaline fermentation (*i.e.,* steered by *Bacillus* spp.) of *Parkia biglobosa* seed kernels and another alternative product explored for *Zamnè* processing (Guissou et al., 2020). Further studies are needed to assess the sensory properties of overripe *Zamnè* tempeh. Meanwhile, the nutritional and flour

technological properties of the products fermented for 48 and 120 h (representing fresh and overripe *Zamnè* tempehs, respectively) were assessed and contrasted in the following sections.

### 3.2. Nutritional properties of the developed fresh and overripe tempehs

The nutritional composition and the digestibility of the precooked *Zamnè* and derived fresh and overripe tempehs, as identified in **section 3.1**, are summarized in **Tables 1** and **2**. The fermentation resulted in significant decreases in the total lipid (by 30%), insoluble dietary fibers (by 22%-56%), and metabolizable energy (by 14%) contents. In contrast, there were significant increases in the contents of the soluble dietary fibers (by 315%) and no considerable change in the crude protein, total dietary fibers, available carbohydrates, crude ash, and the selected minerals (*i.e.*, iron and zinc) contents. Meanwhile, while the fermentation slightly decreased the protein digestibility (by only 4%), it increased more than twice the primary amine contents, indicating a pre-digestion of the proteins by the enzymes produced by the mold (*i.e.*, *R. oryzae*) (Polanowska et al., 2020; Sparringa & Owens, 1999; Stodolak & Starzynska-Janiszewska, 2008). Last but not least, the fermentation did not affect the solubility of either iron or zinc, the most deficient minerals in human nutrition (Hurrell, 2003), but improved twice the dializability of zinc and likely its bioaccessibility. The dialyzability of iron could not be determined due to its undetectable concentration (below the detection limit of 0.03 mg/L) in the dialysates.

The present study illustrates for the first time tempeh production from *Zamnè* and shows the advantage (in terms of nutritional properties) of tempeh fermentation compared to the current processing methods of *Zamnè*. Accordingly, the precooked *Zamnè* in the present study showed comparable nutritional composition and digestibility to the traditionally cooked *Zamnè* (Drabo, 2023). Be that as it may, *Zamnè* tempeh (both fresh and overripe) evokes higher nutritive values (*e.g.*, higher protein hydrolysis degree, zinc bioaccessibility, and soluble dietary fibers) than the traditionally cooked and alkaline fermented (Guissou et al., 2020) *Zamnè*. But still, further investigation will be essential to explore the alkaline fermentation further and to determine the

372 nutritional qualities and technological properties of the dietary fibers, the proteins, and the lipids
373 considering the eventual bioconversion operated by the mold (Ahnan-Winarno et al., 2021).
374 Particularly, it has been reported that the *Rhizopus* genus uses lipids as a primary carbon source
375 and significantly reduces their content and composition during tempeh fermentation (Ahnan-
376 Winarno et al., 2021; Polanowska et al., 2020), requiring more attention. Besides, it is worth
377 highlighting that *R. oryzae* was selected for the present experimental trial due to its ability to
378 produce substantial non-starch or cell wall polysaccharide-degrading enzymes (*e.g.*, xylanase,
379 cellulase, and pectinases) (Ahnan-Winarno et al., 2021; Huang et al., 2019), accounting for the
380 decrease in the insoluble dietary fibers and the increases in the soluble dietary fibers and the
381 available carbohydrates (though minor).

382 In the meantime, the effect of the fermentation on the nutritional composition of *Zamnè* agreed
383 relatively with previous reports on the tempeh fermentation process, as comprehensively
384 reviewed recently by Ahnan-Winarno et al. (2021). However, in line with the present study, a
385 few studies (Jawad et al., 2008) have reported a decrease in protein digestibility and
386 bioaccessibility after tempeh fermentation of African yambean and grasspea. Yet, in the present
387 study, the decrease in the primary amine content after the overripening indicates a decrease in
388 the protein hydrolysis degree (Adler-Nissen, 1979) and could suggest a conversion of the
389 substrates' proteins and amino acids into fungal proteins (Polanowska et al., 2020; Sparringa &
390 Owens, 1999), with lower digestibility and bioaccessibility (Stodolak & Starzynska-
391 Janiszewska, 2008). Considering the different reports (Jawad et al., 2007; Polanowska et al.,
392 2020; Stodolak & Starzynska-Janiszewska, 2008) and the present study, variable factors,
393 including the substrate type, the starter culture choice, and the incubation conditions (*e.g.*, time),
394 could explain the variable effects of non-soybean tempeh fermentation on protein digestibility
395 and bioaccessibility and need further investigation.

Furthermore, only two studies (Kasaoka et al., 1997; Tawali & Schwedt, 1998) have been found reporting the impact of tempeh fermentation on mineral (*i.e.*, iron) solubility and bioavailability (*i.e.*, *in vivo* absorption and use) and showed that soybean tempeh fermentation increases the amount of soluble iron (II) species, decreasing complexed iron and iron (III) species, and thus improve its bioaccessibility and bioavailability. The difference between the previous report on iron bioaccessibility (in soybean tempeh) and the finding in the present study could be explained by the difference in the fermented substrates. Yet, further research is also needed to address this research gap and decipher the mobilization of minerals during tempeh fermentation.

In addition to the effects on the nutritive constituents, tempeh fermentation is reported to variably alter the non-nutritive constituents (*i.e.*, antinutrients and bioactive compounds), such as fibers, phytate, soluble phenolic, and antioxidant compounds of the original substrates (Ahnan-Winarno et al., 2021; Lim et al., 2023; Polanowska et al., 2020). In the present study, the fermentation eliminated phytate to non-detectable levels (LOD = 27.3 mg PAE/100 g dm) and significantly increased the content of the soluble phenolic compounds (by 46%) but decreased their antioxidant activities considerably (by 24%). The elimination of phytate could partly explain the improvement in zinc bioaccessibility (Hurrell, 2003). The contrast between the soluble phenolic contents and the antioxidant activities was surprising but could be due to the transformation (*e.g.*, modification of the glycoside conjugates, oxidation, or polymerization) of the phenolic compounds during fermentation (Ahnan-Winarno et al., 2021; Lim et al., 2023; Polanowska et al., 2020). Further research is needed to understand the effect of tempeh fermentation on the phenolic compounds, considering the transformation of the phenolic compounds of the substrates, the starter culture choice, and the fermentation conditions (*e.g.*, duration, temperature, airflow) (Ahnan-Winarno et al., 2021; Lim et al., 2023; Polanowska et al., 2020).

### 3.3. Impact of the tempeh fermentation on the flour yields and technological properties

The precooking and the fermentation of *Zamnè* significantly improved its flour extractability by 25% and 42%, respectively, and the effects on the appearance and technological properties of the extracted flour were variable (**Fig. 2** and **Table 3**). The colors of the precooked seeds and the tempeh flours were all identified as light brown, while the raw seed flour was identified as straw yellow. However, the flours had comparable bulk density (0.39–0.48 g/mL), similar to wheat and soybean fine flours (Amandikwa et al., 2015; Shevkani et al., 2021) but lighter than flours from most common legumes (*i.e.*, lentils, beans, peas) (0.54–84 g/mL) (Du et al., 2014; Kaur & Singh, 2005; Shevkani et al., 2021).

The impact of the precooking and the tempeh fermentation of *Zamnè* on the derived flours' appearance (*i.e.*, color attributes), physical properties (*i.e.*, bulk density), and technological properties (*i.e.*, WAI, WSI, EA, ES, FA, and FS) were relatively in line with previous findings on tempeh flours from other seeds (including corn, chickpea, and soybean) (Cuevas-Rodríguez et al., 2006; Puteri et al., 2018; Reyes-Moreno et al., 2004). However, all the processed flours exhibited very marginal foaming activities (FA) and foam stabilities (FS) compared to most common legume flours (18%-88% of FA and 7%-47% of FS) but had relatively similar emulsion activity (EA) and emulsion stability (ES) to them and related *Acacia* s.s. seed flours (Adiamo et al., 2021; Du et al., 2014; Shevkani et al., 2021). The low foaming activity and stability of *Zamnè* and *Zamnè* tempeh flours are probably related to the low surface activity of the soluble proteins, the complexation of the proteins with lipids, or the interference of the carbohydrates (*i.e.*, non-starch polysaccharides (**Table 1**)) (Du et al., 2014; Kaur & Singh, 2005; Shevkani et al., 2021), requiring further investigation. Besides, in contrast to the raw seed flour, the precooked seeds and the tempeh flours demonstrated similar water absorption indices (WAI) to the common legumes and related *Acacia* s.s. seeds' flours (Adiamo et al., 2021; Du et al., 2014; Shevkani et al., 2021). The lower WAI of the raw seed flour could be explained by

445 the slow hydration of the raw and intact seed cells, and further investigation is needed to clarify
446 this. Meanwhile, in contrast to the precooked seed flour, the raw seed and the tempeh flours
447 demonstrated similar water solubility indices (WSI) to common legumes and related *Acacia* s.s.
448 seeds' flours (Adiamo et al., 2021; Du et al., 2014). The lower WSI of the precooked seed
449 flower can be explained by the leaching of most of the soluble matter during the precooking
450 process (Drabo et al., 2020).
451 The present study explores for the first time *Zamnè* flours, and it can be concluded, compared
452 with the precooking, that tempeh fermentation could be an effective option for producing flour
453 from *Zamnè* since it improved most of the determined and key technological properties.
454 Moreover, *Zamnè* has been identified as a source of health-beneficial non-starch
455 polysaccharides and cryptic peptides (Patent No. EP 2 506 723 B1, 2017; Zongo et al., 2022).
456 On the other hand, legumes, related *Acacia* seeds, and tempeh flours have received increasing
457 interest in developing healthy food products and nutraceuticals (*e.g.*, healthy beverages,
458 weaning foods, and supplements for older people) (Adiamo et al., 2019; Ahnan-Winarno et al.,
459 2021; Garrido-Galand et al., 2021), making *Zamnè* flour and its tempeh flours potential
460 multifunctional food ingredients. In contrast to tempeh fermentation, it has been reported that
461 fermentations with lactic acid bacteria decrease the water solubility, foaming activity, and
462 emulsion activity of legumes flours (Chandra-Hioe et al., 2016; Pei et al., 2022). However, this
463 should not overshadow many other benefits of legume fermentation with lactic acid bacteria
464 (*e.g.*, the production of exopolysaccharides, the reduction of the antinutritional factors, and the
465 improvement of the flavors, nutritional properties, and sourdough or rheological properties of
466 the flours) (Chandra-Hioe et al., 2016; Pei et al., 2022; Ritter et al., 2022). Further experiments
467 are needed to explore the fermentation of *Zamnè* and might likely include fermentation by
468 bacterial strains (particularly food-grade lactic acid bacteria and *Bacillus* spp).

## 4. Conclusion

This study demonstrated that *Zamnè*, a hard-to-cook, -process, and -digest legume, is fermentable into tempeh and that the fermentation process improves its nutritional properties, particularly the dietary fibers composition (*i.e.*, increase in soluble dietary fibers content and decrease in insoluble dietary fibers content), zinc bioaccessibility, and the protein hydrolysis degree. Moreover, the fermentation has also improved the extractability and technological properties (*i.e.*, water absorption index, water solubility index, and emulsion activity) of *Zamnè* flour, providing a multifunctional food ingredient. Besides, the analysis of the fermentation process enabled the determination of the optimal fermentation time (*i.e.*, 48 h) and determinants (*i.e.*, pH, free ammonia content, CIELAB color indices, and protein hydrolysis degree). As shown, the overripening (up to 120 h of fermentation) of the tempeh product reduced only the total lipid content and the protein hydrolysis degree, requiring further investigation. In contrast, the present study has uncovered intriguing outcomes (*i.e.*, a reduction in the protein digestibility and the discrepancy between the phenolic content and the antioxidant activity) for a non-soybean tempeh fermentation, emphasizing the need for further investigation of non-soy tempehs. Nonetheless, the present study has shown that tempeh fermentation is a promising processing alternative for *Zamnè,* and *Zamnè* fermentation (including the use of bacteria strains) merits further investigation (*e.g.*, formulation of ready-to-eat prototype products, sensory analysis, and dietary trial).

## CRediT authorship contribution statement

**M.S. Drabo**: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft. **A. Savadogo**: Resource, Reviewing and Editing. **K. Raes**: Conceptualization, Resource, Methodology, Data curation, Reviewing and Editing.

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**Table 1**

Nutritional properties of precooked *Zamnè* and derived fresh and overripe tempehs

| Nutritional properties | Precooked *Zamnè*^ | Fresh *Zamnè* tempeh[$] | Overripe *Zamnè* tempeh[£] | ANOVA p-value |
|---|---|---|---|---|
| Moisture (% fw) | 80.8 ±0.6[a] | 76.3 ±1.8[b] | 74.1 ±0.3[c] | <0.001 |
| Lipids (% dm) | 19.1 ±1.2[a] | 13.3 ±0.6[b] | 9.9 ±1.7[c] | <0.001 |
| Proteins (4.05 x Kjeldahl Nitrogen) (% dm) | 30.9 ±1.3[a] | 33.1 ±0.6[a] | 34.1 ±2.0[a] | 0.053 |
| % of digestible proteins | 76.9 ±1.2[a] | 74.0 ±0.3[b] | 73.8 ±0.7[b] | <0.001 |
| Primary amines (mmol/100 g protein) | 99.5 ±4.2[a] | 254 ±36[b] | 175 ±26[a] | 0.002 |
| Total carbohydrates (% dm) | 46.8 ±2.9[a] | 50.6 ±0.7[a] | 53.2 ±0.6[a] | 0.079 |
| Available carbohydrates (% dm) | 1.9 ±0.1[a] | 2.8 ±0.6[ab] | 3.6 ±0.8[b] | 0.005 |
| Total dietary fibers (% dm) | 44.9 ±2.7[a] | 48.1 ±0.1[a] | 50.2 ±0.7[a] | 0.102 |
| Insoluble dietary fibers (% dm) | 40.8 ±2.2[a] | 31.9 ±3.0[b] | 34.6 ±1.1[b] | 0.007 |
| Soluble dietary fibers (% dm) | 4.0 ±3.5[a] | 15.6 ±3.4[a] | 15.2 ±1.7[a] | 0.078 |
| Metabolizable energy (kcal/100 dm) | 394 ±12[a] | 359 ±1[ab] | 339 ±10[b] | 0.018 |
| Ash (% dm) | 2.8 ±0.2[a] | 2.9 ±0.2[a] | 2.7 ±0.5[a] | 0.765 |
| Zinc (mg/100 dm) | 7.8 ±0.3[a] | 8.4 ±0.5[a] | 7.9 ±0.2[a] | 0.164 |
| Iron (mg/100 dm) | 3.5 ±0.4[a] | 4.1 ±0.2[a] | 3.6 ±0.4[a] | 0.162 |
| Soluble phenolics (mg GAE/100 g dm) | 396 ±44[a] | 578 ±76[b] | 481 ±37[ab] | 0.004 |
| Antioxidant activity (mg TE/100 g dm) | 471 ±64[a] | 356 ±51[b] | 332 ±42[b] | 0.011 |

The raw seeds had 4.8 g/100 g fresh weight (fw) of moisture, undetectable starch content (detection limit = 0.63 g glucose equivalent/100 g dry matter (dm)), 12.5 g of total lipid, 18.1 g of total protein, 16.3 g of crude fiber, and 3.8 g of ash (per 100 g dm). ^ The precooked seeds included the starter but were frozen at time 0 h. [$, £] The precooked *Zamnè* is fermented for 48 and 120 h using *Rhizopus oryzae*, respectively. GAE and TE = gallic acid and trolox equivalents, respectively. The values in the same row with the different letter superscripts are significantly different ($p < 0.05$, Tukey-Kramer rank test, and n = 2 product batches x 2 assay replicates).

**Table 2**

Bioaccessibility of minerals and total phytate content in precooked *Zamnè* and derived fresh and overripe tempehs

| Assay[Δ] | Precooked *Zamnè*[^] | Fresh *Zamnè* tempeh[$] | Overripe *Zamnè* tempeh[£] | ANOVA p-value |
|---|---|---|---|---|
| Solubility (%) | | | | |
| Zinc | 23.5 ±13.4[a] | 13.5 ±3.4[a] | 15.4 ±9.7[a] | 0.499 |
| Iron | 37.9 ±12.2[a] | 31.1 ±8.3[a] | 30.8 ±10.4[a] | 0.714 |
| Dialyzability (%)* | | | | |
| Zinc | 3.4 ±0.3[a] | 9.3 ±0.6[b] | 7.6 ±0.5[b] | <0.001 |
| Phytate (mg PAE/100 g dry matter) | 533 ±52 | <LOD | <LOD | |

[^] The precooked seeds included the starter but were frozen at time 0 h. [$, £] The cooked Zamnè is fermented for 48 and 120 h using *Rhizopus oryzae*, respectively. *The content of iron in the dialysates was below the quantification limit (0.03 mg/L) and could not enable the calculation of its dializability. <LOD = below the detection limit (27.3 mg phytic acid equivalent (PAE)/100 g dm). The values in the same row with the different letter superscripts are significantly different ($p < 0.05$, Tukey-Kramer rank test). [Δ]n = 2 product batches x 2 assay replicates, except for the phytate content determination, where n = 2 product batches x 3 assay replicates.

**Table 3**

Yield and technological properties of *Zamnè* flours as affected by precooking and tempeh fermentation

| Pre-processing before the flour extraction | Raw *Zamnè* | Precooked *Zamnè* | Fresh *Zamnè* tempeh[$] | Overripe *Zamnè* tempeh[£] | ANOVA p-value |
|---|---|---|---|---|---|
| Flour yield (%) | 50.4 ±2.3[a] | 75.7 ±0.1[b] | 93.6 ±7.4[c] | 91.6 ±0.5[c] | 0.001 |
| Color perception[ǂ] | Straw | Light brown | Light brown | Light brown | |
| L* | 76.3 ±0.5[a] | 54.4 ±2.9[b] | 54.8 ±3.1[b] | 53.0 ±0.2[b] | 0.001 |
| a* | 1.9 ±0.1[a] | 6.8 ±0.2[bc] | 6.4 ±0.2[b] | 7.4 ±0.1[c] | <0.001 |
| b* | 30.7 ±2.1[a] | 25.0 ±1.1[ab] | 23.0 ±2.1[b] | 24.1 ±1.0[a] | 0.032 |
| Whiteness index | 61.1 ±1.3[a] | 47.5 ±2.1[b] | 48.8 ±1.9[b] | 46.7 ±0.3[b] | 0.002 |
| Chroma value | 30.8 ±2.1[a] | 25.9 ±1.0[ab] | 23.9 ±1.9[b] | 25.2 ±1.0[ab] | 0.044 |
| Bulk density (g/mL) | 0.48 ±0.08[a] | 0.39 ±0.14[a] | 0.39 ±0.10[a] | 0.42 ±0.09[a] | 0.850 |
| Water absorption index (g/g) | 2.9 ±0.3[a] | 5.2 ±0.5[b] | 4.2 ±0.4[ab] | 4.5 ±0.2[b] | 0.013 |
| Water solubility index (%) | 35.0 ±1.2[a] | 8.6 ±0.6[b] | 22.9 ±0.7[c] | 23.5 ±0.6[c] | <0.001 |
| Emulsion activity (%) | 45.9 ±3.4[a] | 53.1 ±0.1[ab] | 58.0 ±3.4[b] | 60.4 ±0.1[b] | 0.014 |
| Emulsion stability (%) | 100 ±0[a] | 97.7 ±3.2[a] | 98.0 ±2.8[a] | 98.0 ±2.8[a] | 0.801 |
| Foaming activity (%) | 27.8 ±3.9[a] | 9.7 ±5.9[a] | 26.9 ±6.6[a] | 19.4 ±7.9[a] | 0.124 |
| Foaming stability (%) | 75.2 ±3.6[a] | 15.0 ±21.2[a] | 45.8 ±29.5[a] | 32.2 ±17.3[a] | 0.146 |

[$, £] The cooked *Zamnè* is fermented for 48 and 120 h using *Rhizopus oryzae*, respectively. [ǂ]CIELAB color space values L* (brightness), a* (green-red opponents), and b* (blue-yellow opponent) were converted to hex color codes and then color names by using R package colorspace. The values in the same row with different letter indices (a-f) are significantly different ($p < 0.05$, Tukey-Kramer rank test. n = 2 product batches x 1 assay replicates.

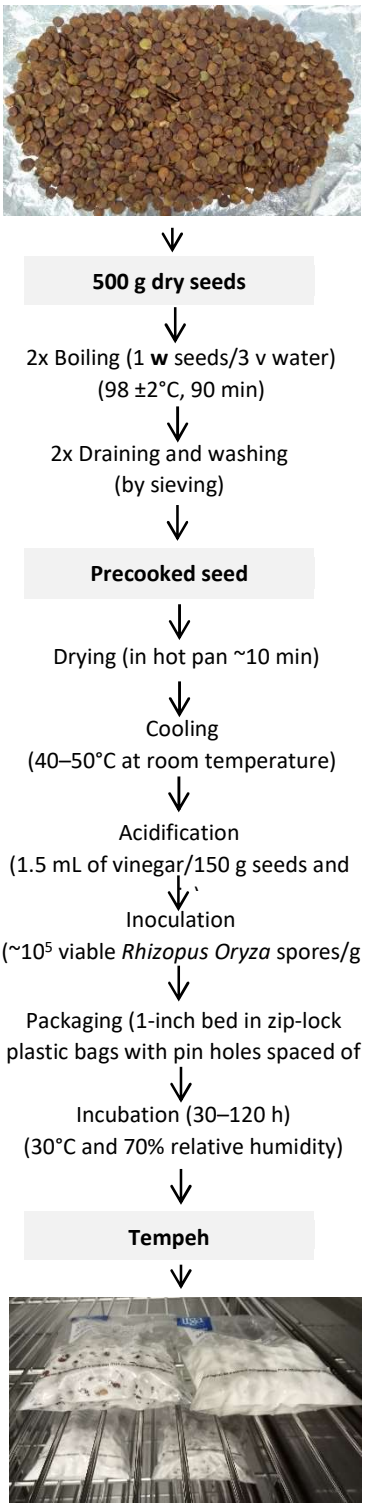


**Fig. 1**. Production diagram of *Zamnè* tempeh (adapted from Ahnan-Winarno et al. (2021))

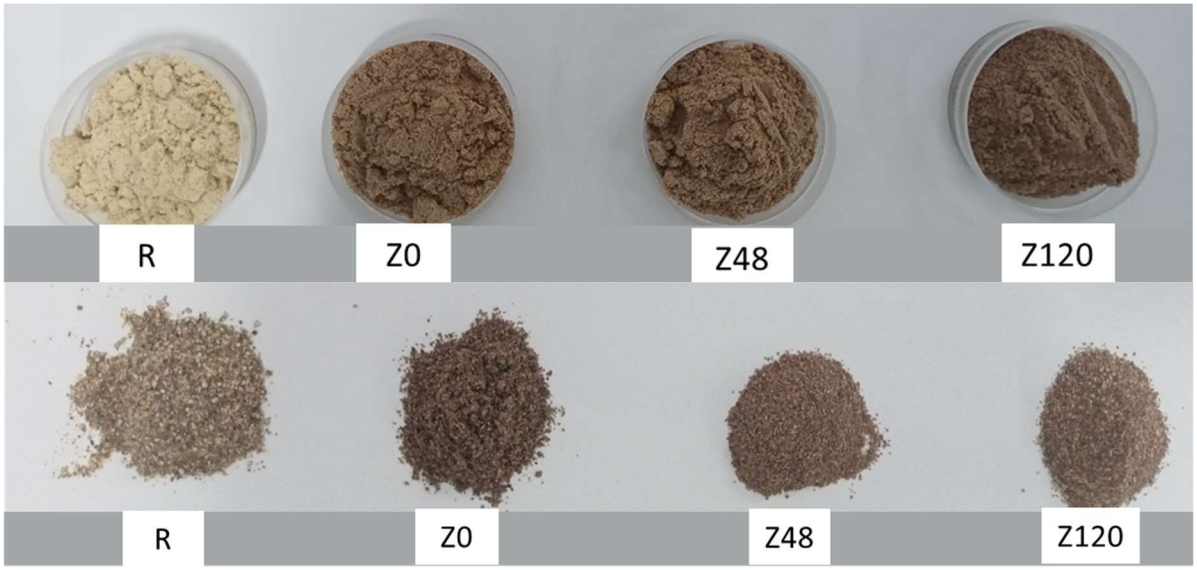


**Fig. 2.** Illustration of the flours and brans from *Zamnè* raw seeds (R), precooked seeds (Z0), fresh tempeh (Z48), and overripe tempeh (Z120)

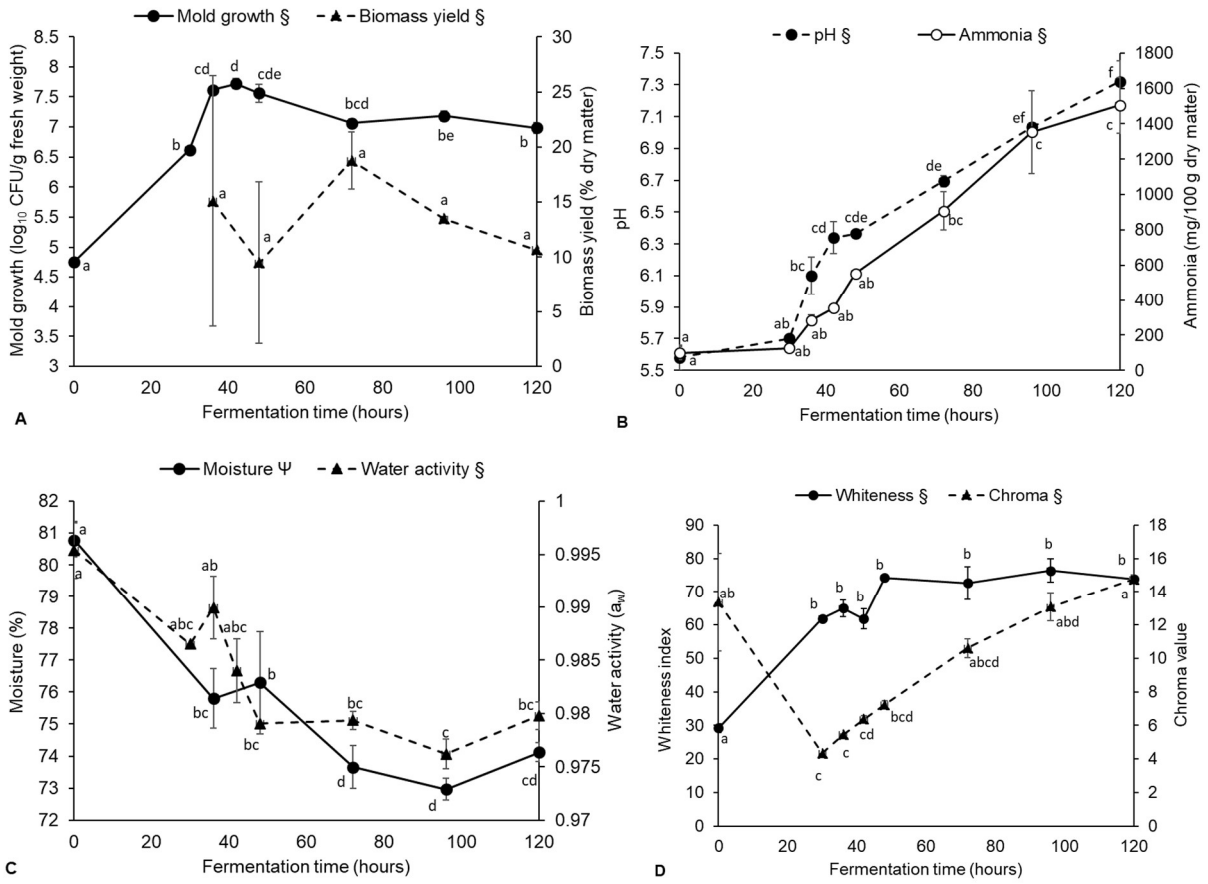


**Fig. 3.** The mold growth and physicochemical changes during the development of *Zamnè* tempeh

CFU = colony-forming unit (of *Rhizopus oryzae*). The biomass yield after 30 h of fermentation was not determined. The values of the same parameter with different letter indices (a-f) are significantly different ($p < 0.05$, Tukey-Kramer rank test). $^{\S}n = 2$ product batches x 1 assay replicate and $^{\Psi}n = 2$ product batches x 3 assay replicates.

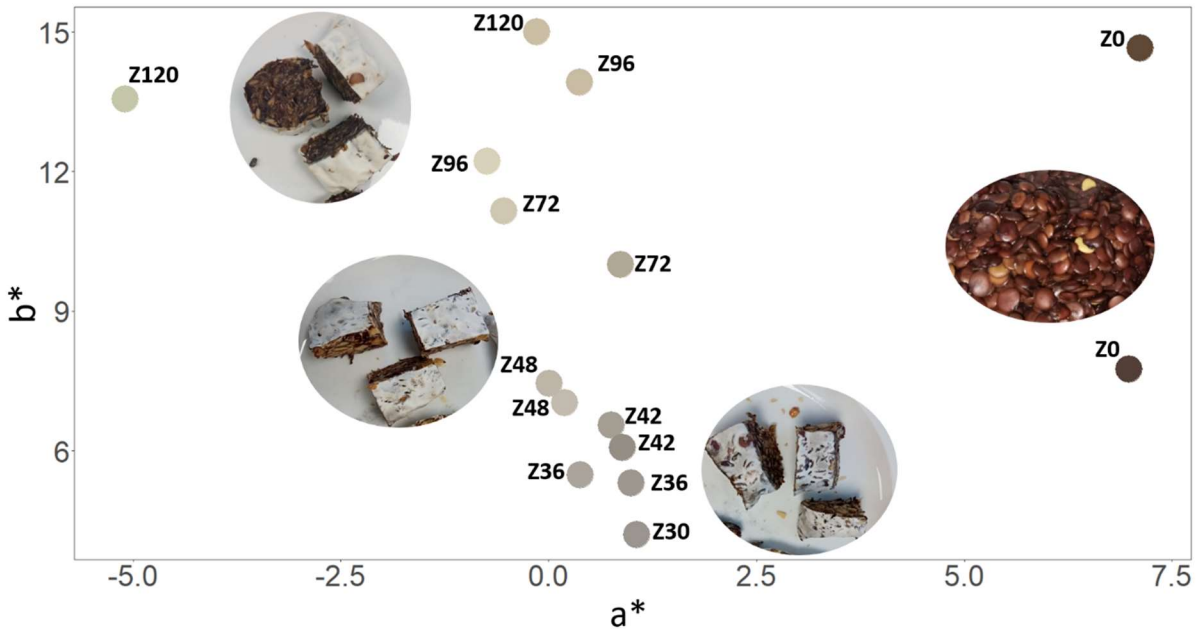


**Fig. 4.** Illustration of the products

CIELAB color space values L* (brightness), a* (green-red opponents), and b* (blue-yellow opponent) were converted to hex color codes and displayed on a* and b* dimensions by using R package colorspace. Z0-120 = precooked and fermented *Zamnè* into tempehs (the number subscripts indicate the fermentation times in hours). The products Z0, Z36, Z48, and Z120 are illustrated. The duplicate of Z30 did not give a well-compacted cake and was then not measured.