Microbiology of fermented soy foods in Asia: can we learn lessons for production of plant cheese analogues?

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Abstract

The food industry is facing the challenge of creating innovative, nutritious, and flavored plant-based products, due to consumer's increasing demand for the health and environmental sustainability. Fermentation as a unique and effective tool plays an important role in the innovation of food products. Traditional fermented soy foods are popular in many Asian and African countries as nutritious, digestible and flavorful daily staples or condiments. They are produced by specific microorganisms with the unique fermentation process in which microorganisms convert the ingredients of whole soybean or soybean curd to flavorful and functional molecules. This review provides an overview on traditional fermented food produced from soy, including *douchi*, *natto*, *tempeh*, and *sufu* as well as stinky tofu, including the background of these products, the manufacturing process, and the microbial diversity involved in fermentation procedures as well as flavor volatiles that were identified in the final products. The contribution of microbes to the quality of these five fermented soy foods is discussed, with the comparison to the role of cheese ripening microorganisms in cheese flavor formation. This communication aims to summarize the microbiology of fermented soy foods in Asia, evoking innovative ideas for the development of new plant-based fermented foods especially plant-based cheese analogues.

Keywords. douchi, natto, tempeh, sufu, plant-based cheese, *Lactobacillus, Bacillus, Aspergillus*.

Introduction

Fermentation is a traditional, low cost and effective unit operation in food production. It transforms agricultural raw materials into functional and flavored foods through desirable microbial growth and enzymatic activity (Marco et al., 2021). Fermentation is also considered as an effective tool to improve food safety through the reduction of anti-nutritive or even toxic compounds, and through fermentative production of antimicrobial products including organic acids, alcohols, and bacteriocins by that control spoilage organisms and pathogens (Gänzle, 2020; Ross et al., 2002). The characteristics and quality of final fermented products depends on the dominant microorganisms or core microbiota. Microorganisms commonly used for food fermentation include lactic acid bacteria, acetic acid bacteria, yeasts and filamentous fungi (Marco et al., 2021). Globally, fermented protein foods are prepared from soy or legumes, or from animal protein. In the European tradition of food fermentations, fermented protein foods are exclusively produced with animal proteins, i.e. meat, milk or fish (Gänzle, 2022). The current trend towards replacing animal proteins with plant proteins to increase the sustainability of food production, and to meet the demand of vegan or vegetarian consumers also necessitates to develop plant-based analogues of fermented animal protein foods (Grossmann and McClements, 2021). To date, the development of these fermented plant-protein foods was informed by the traditional use of cultures in meat and cheese fermentations, or the selection of starter cultures defaults to *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* (Mefleh et al., 2021). However, traditional fermentation of plant protein foods, particularly fermented foods prepared from soy beans, may provide an additional knowledge base for design of fermentation processes, and for selection of starter cultures.

Legumes are among of the most important agricultural crops. Multiple traditional fermented foods employ legumes as main substrate and, among legumes, soy beans are most commonly used as substrate for traditional fermented protein foods (Gänzle, 2022). Soy beans were domesticated several thousand years ago in East Asia and has been introduced to other parts of the world less than 500 years ago. It has a high protein content, about ~ 40% and a well-balanced amino acid composition (Etten et al., 1959; Medic et al., 2014). In addition, soy proteins exhibit good functional properties, including gelling formation, emulsification, water-holding, and foaming (Wolf, 1970). Thus, soybeans are an important source of plant proteins. In addition to the high content of proteins, soybeans also contain other nutrients including diverse carbohydrates and lipids as well as isoflavones (Medic et al., 2014). In East Asian countries, a variety of fermented soy foods has been traditionally prepared and consumed as staple foods or as condiments; examples include *douchi*, *natto*, *tempeh*, and *sufu*. Of these, *douchi*, *natto*, and *tempeh* are whole soybean-fermented products while *sufu*, a soft creamy "cheese-like" product, is fermented soybean curd (Lite, 2005).

Fermented soy foods are produced with diverse fermentation microbes. *Natto* is fermented with *Bacillus subtilis* biovar. Natto (Hosoi and Kiuchi, 2008), but *Rhizopus* spp. is a common starter culture for tempeh-making (Hartanti et al., 2015). Traditional fermented soy foods such as *douchi*, *sufu*, and *tempeh* are made using spontaneous fermentation, or with pure starter cultures to initiate the fermentation, followed by the maturation (Han et al., 2001b; Lite, 2005; Nout and Rombouts, 1990). Their microbial communities significantly differ depending on the processing method even though they are the same type of fermented soy products, subsequently influencing the flavor and quality of final products. There are two *douchi* products, *Aspergillus*-type Liuyang *douchi* and *Mucor*-type Yongchuan *douchi*, that exhibit remarkable differences in the microbiota composition and flavor (Yang et al., 2019; Zhang et al., 2021). Although the fermentation process, microbial compositions and their function on the flavor formation have been described, there are few studies on summarizing their microbial diversity and microbial contributions to the flavor and quality of final fermented soy products.

The review summarizes the production process and microbiology of five fermented soy foods in Asia, including *douchi, natto, tempeh*, and *sufu* as well as stinky tofu, evaluates the potential contribution of microbes to the flavor and quality of final products, and discusses the question whether these traditional fermented products provide guidance for the development of plant-based analogues of plant-based cheeses and fermented meats.

Fermentation of soy beans.

Douchi

Douchi, a traditional fermented soybean product has been consumed in China as a protein source or seasoning, and has been used in Chinese traditional medicine (Chen et al., 2007; Lite, 2005; Lite et al., 2002). The production of *douchi* involves a two-stage fermentation process: koji-making or pre-fermentation and maturation or post-fermentation (Figure 1). According to the microbes in the koji-making phase, *douchi* can be grouped into three types: *Aspergillus*-type, i.e. Baoxiangyuan and Liuyang *douchi*, *Mucor*-type, i.e. Yongchuan *douchi*, and *Bacillus*-type, i.e. Qingyang *douchi and* Longnan *douchi* (Chen et al., 2007; Lite, 2005; Zhang et al., 2018). *Mucor douchi* is fermented at a lower temperature but for a longer fermentation time when compared to *Aspergillus* type *douchi* (Figure 1) (Lite, 2005). The salt content of *Aspergillus*-type Baoxiangyuan and Liuyang *douchi* ranges from 4 to7% (w/w) (T. Chen et al., 2011b; Yang et al., 2016; Zhang et al., 2022), which are low-salt *douchi* products, but other *Aspergillus*-type *douchi* is produced with up to 16% (w/w) NaCl (Lite, 2005; Zhang et al., 2007). *Mucor*-type *douchi* is commonly produced as high-salt *douchi* with a salt content of more than 12% (He et al., 2016; Hu et al., 2012).

Aspergillus-type *douchi* is most widely produced. *Aspergillus*-type *douchi* is made in Southern China, e.g. Hunan, Jiangxi, Sichuan, and Guangdong provinces (Q. C. Chen et al., 2011; Chen et al., 2012; Fan et al., 2009). *Aspergillus* spp. and *Bacillus* spp. were identified as dominant microorganisms during pre-fermentation; *Aspergillus* strains originate from inoculation with starter cultures while *Bacillus* spp. originate from the 'house flora' or the raw material (T. Chen et al., 2011a, 2011b; Zhang et al., 2007). In the pre-fermentation step, *Aspergillus* and *Bacillus* spp. produce hydrolytic enzymes including proteases, cellulases, amylases, and lipases and thus generate substrates for other microorganisms in the subsequent *douchi* maturation (Hu et al., 2022; Pel et al., 2007; Zhang et al., 2007). To prevent the formation of bitterness and astringency of the final products, the extend of hydrolysis is controlled by washing and addition of brine (Lite, 2005). *Douchi* is subsequently maturated in a brine mixture of salts and seasonings without addition of starter cultures; the microbial communities of *douchi* and the flavor of the endproducts varies depending on the change of environmental conditions. The bacterial and fungal communities listed in Table 1 were identified as the dominant microbiota during maturation of *Aspergillus*-type *douchi* or in mature Baoxiangyuan and Liuyang *douchi*, which contain 4 – 7% NaCl (Chen et al., 2012, 2014, 2022; T. Chen et al., 2011b; Li et al., 2018; Yang et al., 2019, 2016).

Mucor-type *douchi* is mainly produced in South-Western China (Wang et al., 2021; Yu et al., 2022; Zhang et al., 2021) In pre-fermentation, *Mucor* species are inoculated as starter cultures (Lite, 2005) and also secrete hydrolytic enzymes. *Mucor qu* is prepared at lower temperature with slower fermentation compared to *Aspergillus qu* (Figure 1). For *Mucor*-type *douchi*, *Mucor qu* is mixed directly with salts and seasonings without washing, followed by the secondary fermentation. During post-fermentation phase of Yongchuan *douchi*, the bacterial genera *Bacillus*, *Enterobacter*, and *Pseudomonas*, and the fungal genera *Penicillium* and *Aspergillus* were identified as dominant members of fermentation microbiota (Zhang et al., 2021).

Bacteria-type *douchi* is commonly produced in North China including Beijing and the Shandong and Gansu provinces. It is usually generated through the spontaneous fermentation with “house flora”. Thus, the specificities of traditional bacteria-type *douchi* production vary depending on the environmental conditions. Zhang et al. (Zhang et al., 2018) characterized the microbial diversity of two bacteria-type *douchi* products and found that the microbial communities differed. The bacterial genera *Bacillus* and *Ignatzschineria* and the fungal genera *Pichia* and *Candida* were present in both *douchi* products.

The accumulation of tastants and volatile odorants imparts fermented *douchi* with a unique odor and taste. The content of free amino acids increased gradually during fermentation (Zhang et al., 2007) and most of the volatiles are generated in the post-fermentation stage (Yang et al., 2019). Because the optimum water activity for the Maillard reaction, 0.5 - 0.7 (Pereyra Gonzales et al., 2010), matches the water activity of *douchi* during drying, Maillard products that are formed during drying may additionally contribute to the flavor formation of *douchi* (L. Liu et al., 2022). Different types of *douchi* exhibit a different composition of volatiles due to the distinct composition of fermentation microbiota during post-fermentation. In *Aspergillus*-type Liuyang *douchi*, the volatiles phenylethyl alcohol, phenethyl acetate, isoamyl acetate, 2-methyl-butanal, ethyl 2-methylbutyrate, 2,6-dimethylpyrazine, 1-octen-3-ol, 2-pentyl furan, benzeneacetaldehyde and phenethyl butyrate were identified (Q. C. Chen et al., 2011; Chen et al., 2021). Naturally maturated *Mucor*-type *douchi* contained a higher diversity of volatile compounds including esters, phenols, and pyrazines compared to *Mucor*-type *douchi* inoculated by defined starters (He et al., 2016), likely reflecting that more diverse fermentation microbiota generally result in a more diverse and intense flavor profile. The use of co-cultures which consist of the salt tolerant *Tetragenococcus halophilus*, *Zygosaccharomyces rouxii* and *Candida versatilis* accelerated the formation of volatiles (He et al., 2016). Wang et al. (Wang et al., 2021) identified 10 compounds present with high odor activity values in *Mucor*-type Yongchuan *douchi*, including diacetyl, dimethyl trisulfide, acetic acid, acetylpyrazine, 3-methylvaleric acid, 4-methylvaleric acid, 2-methoxyphenol, maltol, γ-nonanolactone, eugenol and phenylacetic acid. *Aspergillus*-type *douchi* had more phenols and pyrazines while *Mucor*-type *douchi* contained more esters and acids (Wang et al., 2010b). *Mucor*-type Yongchuan *douchi* matured for 10 month contained a higher content of the umami-tasting glutamic acid and aspartic acid and a lower content of bitter-tasting amino acids such as isoleucine, leucine and arginine when compared to *Aspergillus*-type Yongchuan *douchi* that was matured for one month (Zhang et al., 2021). Esters that impart floral and fruity odors, including ethyl isovalerate, isoamyl acetate, and ethyl phenylacetate, were also present in higher quantities in the *Mucor*-type Yongchuan *douchi* when compared to *Aspergillus*-type Yongchuan *douchi* (Zhang et al., 2021).

Natto

Natto, a Japanese fermented soybean food, is categorized into three types based on the production methods and microorganisms used, including *yukiwari-natto*, *hama-natto,* and *itohiki-natto*. *Itohiki-natto* (hereafter shortened to “natto”) is fermented with *Bacillus subtilis* biovar. Natto and is the most widely consumed product in Japan (Hosoi and Kiuchi, 2008; Reddy et al., 1983; Ruiz Sella et al., 2021). The procedure for *natto*-making is shown in Figure 2. Natto strains are able to impart *natto* with the characteristic flavor and the sticky texture after fermentation. They have a very similar genome structure to other *B. subtilis* strains that are not suitable for *natto*-making but additionally require biotin for growth (Kubo et al., 2011). The sticky material on the *natto* surface consists mainly of poly-γ-glutamate (γ-PGA) and polysaccharides (Saito et al., 1974). The texture of *natto* is dependent on γ-PGA; the amount of γ-PGA produced, in turn, relates to the available carbon sources and the concentration of L-glutamate (Shih and Van, 2001). More γ-PGA was produced by *Bacillus subtilis* biovar. Natto using a medium with citric acid, ammonium sulfate and a lower concentration of L-glutamate (Kunioka, 1995). The genetic determinants of *Bacillus* species that relate to their use in food fermentations including *natto* fermentation were recently reviewed (Li et al., 2023).

The flavor characteristics of *natto* depends on the fermentation conditions, the raw material, and *natto* strains that are used (Wei et al., 2001). A storage temperature of more than 15 °C promoted the production of ammonia and branched-fatty acids during maturation, leading to an undesirable ammonia smell (Kanno and Takamatsu, 1987). Thus, storage temperatures of less than 10 °C are used to obtain good quality *natto* (Kada et al., 2008). Soybeans with a higher sugar content but a lower protein and oil content generated *natto* products with superior sensory properties (Yoshikawa et al., 2014). Natto strains secret protease, especially the serine protease nattokinase, to hydrolyse proteins into peptides and amino acids (Ichishima et al., 1986; Ju et al., 2019; Lan et al., 2020). Natto strains also express levansucrase to synthesize the polysaccharide levan from sucrose or raffinose (Bersaneti et al., 2018; Shih et al., 2010, 2005). Pyrazines produced by *B. subtilis* biovar. Natto strains are key contributor to the characteristic odour of natto (Kłosowski et al., 2021; Kosuge et al., 1971; Liu et al., 2018a; Sugawara et al., 1985). Key aroma compounds in natto include 2,3-butanedione, 5-methyl-2-hexanone, 3-hydroxy-2-butanone, 2-nonanone, furaldehyde, acetic acid, 2-ethyl butyric acid, ethyl acetate, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 3,5-dimethyl-2-ethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3,5-methyl-6-

ethylpyrazine, and benzaldehyde; of these, the pyrazines exhibited the highest odor activity values (Liu et al., 2018b; Tanaka et al., 1998).

Tempeh

Tempeh (or *tempe*) is a mold-fermented soybean food which is commonly consumed in Indonesia as snack or as staple food (Nout and Rombouts, 1990). Other legumes, cereal grains and processing by-products have been also used for the tempeh production. Tempeh-making involves a two-stage fermentation: the natural fermentation by lactic acid bacteria during soybean soaking and fungal fermentation by *Rhizopus* spp. starter cultures (Figure 3). In the first-stage, spontaneous fermentation, microorganisms shown in Table 2 were identified, in which *Lacticaseibacillus casei*, *Enterococcus faecium* and *Staphylococcus epidermidis* are dominant microbiota responsible for the acidification during the soaking of soybeans, where the pH value drops from 6.5 to 4.5 (Mulyowidarso et al., 1989). The to increase the reliability of the acidification by lactic acid bacteria during soaking, the addition of lactic or acetic acids to the soaking water (Nout et al., 1985), back-slopping of the soaking water, or use of *Lactiplantibacillus plantarum* as starter culture were used (Nout et al., 1987b). *Leuconostoc fallax, Pediococcus pentosaceus*, and *Weissella cibaria* were identified as dominant members of bacterial communities after 10 back-slopping cycles at 30 °C (Yan et al., 2013). Acidification to pH values ranging from pH 3.5–5.0 (Yoneya, 2004) inhibits the growth of potential pathogenic and spoilage microorganisms including *Staphylococcus aureus* (Nout et al., 1988) and *Bacillus cereus* (Nout et al., 1987a) but does not impact growth of *Rhizopus* if the pH remains above 3.5.

After soaking, the hulls of the soybeans are removed, followed by a cooking process that softens the matrix and eliminates most undesirable microorganisms. In the second-stage of fermentation, *Rhizopus* spp. such as *R. arrhizus*, *R. delemar*, *R. microsporus* and *R. stolonifer* are dominant

(Dwidjoseputro and Wolf, 1970; Hartanti et al., 2015; Sjamsuridzal et al., 2021). In addition to *Rhizopus* spp, bacteria and yeasts have frequently been isolated from tempeh (Table 2) (Ashenafi and Busse, 1991a; Mulyowidarso et al., 1990; Samson et al., 1987). The use of co-inoculant lactobacilli in tempeh fermentation prevents the growth of undesirable microorganisms and generate a high quality product (Nout et al., 1987a). *Citrobacter freundii*, *K. pneumoniae* or *Propionibacterium freudenreichii* have also been investigated as a co-inoculants to increase the yield of vitamin B12 in tempeh fermentation (Keuth and Bisping, 1994; Signorini et al., 2018; Wiesel et al., 1997).

Fresh tempeh appears as a firm cake covered with white mycelium and a meaty, mushroom-like and nutty flavor (Hachmeister and Fung, 1993). *Rhizopus* spp. strains as dominant fermenters produce a variety of glycosyl hyrolases, proteases and lipases to break down polysaccharides, proteins and lipids (Nout and Rombouts, 1990). Amino acids and peptides impart umami taste (Amin et al., 2020). 2-Methylpropanal, 1-octene-3-ol and 3-(methylthio)propanal were determined as the major aroma compounds in tempeh fermented by *R. oligosporus* NRRL 2710 (Jeleń et al., 2013). The use of the co-inoculant *Saccharomyces cerevisiae* additionally contributed to the formation of alcohol, ester, styrene, phenol, and maltol compared to the regular fermented tempeh (Kustyawati et al., 2017).

Fermentation of precipitated soy proteins

Sufu (furu)

Sufu (*Furu* or *Dou-furu*), a fermented soybean curd product is a soft, flavoured and creamy cheese-like product that is consumed as staple food in China. The production of *sufu* involves two-step fermentation processes: pre-fermentation or *pehtze*-making and post-fermentation or *sufu* ripening

(Figure 4). According to the microbes that are present in *pehtze*-making, *sufu* can be grouped into mold-fermented and bacteria-fermented sufu (Han et al., 2001b).

Dominant microorganisms during *sufu* ripening are shown in Table 3. The molds *Actinomucor* spp., *Mucor* spp., and *Rhizopus* spp. are commonly used as pure starter cultures for the *pehtze*-making of mold-fermented sufu (Cheng et al., 2011; Han et al., 2001b, 2004a; Huang et al., 2018; X. Li et al., 2021), which produce proteases (Chou and Hwan, 1994; Han et al., 1988). Strains of *Micrococcus* spp. and/or *Bacillus* spp are also used as starter cultures for the production of bacteria-fermented sufu (Bao et al., 2020; Han et al., 2004a), of these two, *Bacillus* spp. produce extracellular proteases (Li et al., 2023).

Flavor formation occurs predominantly in the ripening stage of *sufu* fermentation and is highly dependent on the dressing mixtures (Figure 4) and the activity of microorganisms (He et al., 2022; He and Chung, 2020; Huang et al., 2018; Song et al., 2021; Yao et al., 2021). The final salt content of *sufu* ranges from 10 to 14%, imparting a salty taste to *sufu* (Han et al., 2001b). In ripened *sufu*, free amino acids accumulate and umami-tasting glutamic acid was the most abundant (Han et al., 2004b; Kim et al., 2011; Li et al., 2010; Xie et al., 2018; Yao et al., 2021). Ethyl 2-methylpropanoate, diacetyl, ethyl butanoate, ethyl 2-methylbutanoate, 3-(methylthio)propanal, ethyl 3-phenylpropionate, 2-heptone, ethyl pentanoate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, benzaldehyde, and phenylethanol contribute to the aroma of red sufu (Chung, 2000; Chung et al., 2005; Wang et al., 2019). (E,E)-2,4-Decadienal, ethyl hexanoate, eugenol, methional, ethyl 2-methylbutyrate, (E,E)-2,4-nonadienal, and 1-octen-3-ol were identified as aroma compounds with high odour activity value in white *sufu* (He et al., 2020). Production of volatile compounds including hexadecenoic acid ethyl ester, methoxy acetic acid pentyl ester, benzene propanoic acid ethyl ester, ethyl 9-hexadecenoate, ethyl oleate, ethanol, 3-methyl-1-butanol, 5-

methoxy-1-pentanol, and eugenol during ripening provided the typical flavor for bacteria-fermented Kedong *sufu* (Fan et al., 2019).

Stinky tofu

Stinky tofu (*choudoufu*), a popular Chinese snack, essentially consists of tofu seasoned with a fermented brine. The flowchart for making of stinky tofu is shown in Figure 5. The Mandarin word for stinky tofu (臭豆腐, *choudoufu*) is best translated as “smelly tofu” because the word “*chou”* has no negative connotation and is also used for strong but acceptable flavors. In contrast, the English word “stinky” is used to describe a negative perception of odor. The scientific literature, however, consistently refers to *choudoufu* as “stinky tofu” so this communication follows convention. The brine with the strong odour is produced by fermentation. Two formulas with different raw materials are commonly used, one consisting mainly of mixed vegetables such as spiny amaranth, bamboo shoot and wax gourd with or without meat; the second additionally contains mashed tofu (Chao et al., 2008). Only few studies describe differences in the microbial communities in the two types of stinky brines. Also, microbes that are present during different fermentation phases are only partially investigated (Gu et al., 2018a). Lactic acid bacteria and the genus *Bacillus* are considered as dominant microbiota in the fermentation of stinky brines. Organisms of the genera *Enterococcus*, *Lactococcus*, *Streptococcus,* several genera of the *Lactobacillaceae* as well as *Bacillus* spp. such as *B. megaterium*, *B. polymyxa*, *B. pumilus*, *B. subtilis,* and *B. sphaericus* were identified in fermented stinky brines (Chao et al., 2008; 2010; Gu et al., 2018b; Lei et al., 2013; G. Liu et al., 2022). The lactic acid bacteria in stinky tofu brines overlap with core microorganisms in spontaneous vegetable fermentation (Ashaolu and Reale, 2020). The strains of the genus *Paraeggerthella* and *Eggerthella* involving the S-equol production were also isolated from brines of stinky tofu (Abiru et al., 2013).

Stinky tofu is categorized as alkaline fermented food where ammonia is produced from amino acids during fermentation. Ammonia formed during the fermentation of stinky brines results from the deamination of amino acids in neutral or alkaline conditions. Ammonia and CO_2 in the stinky brine permeates into the tofu during brining but evaporates during deep-frying or steaming process, imparting a fluffy and porous texture on the surface of stinky tofu (Teng et al., 2004). Volatile compounds imparting the characteristic flavor of stinky brines include camphene, caryophyllene, D-limonene, 3-(1,5-dimethyl-4 hexene)-6 methylene-cyclohexene, 5-(1,5-dimethyl-4-hexene)-2-methyl-1,3-cyclohexane), butanoic acid, pentanoic acid, heptanoic acid, octanoic acid, nonanoic acid, N-decanoic acid, dodecanoic acid, tridecanoic acid, hexadecenoic acid, and tetradecanoic acid, 2,6-dimethyl-2-octanol, butane, pentanoic acid-2,2,4-trimethyl-3-carboxyisopropyl-isobutyl ester, 2-undecanone, dimethylamine, and sulfur hydrogen sulfide (Wang et al., 2020).

Similarities and difference of fermented soy products and cheeses.

Focussing on the differences first, the substrate supply in soy or plant-based cheese analogues produced from soy or pulses along with other ingredients such as nuts or seeds and fats or oils differs substantially from the substrate supply in milk or cheeses. Milk contains lactose as sole source of fermentable carbohydrates and lactose metabolism is a major criterion for starter cultures (van de Guchte et al., 2006; Wels et al., 2019). Soybean and soybean curd contain more diverse carbohydrates, including fructose, glucose, sucrose, maltose, raffinose, stachyose and verbascose (Medic et al., 2014) and the ability of cultures to ferment raffinose-family oligosaccharides likely impacts acidification (Teixeira et al., 2012). In addition, plant-based cheese analogues contain starch and non-starch polysaccharides (Medic et al., 2014), which are completely absent in milk or cheese. Extracellular glycosyl hydrolases that degrade starch and non-starch polysaccharides thus impact the ripening. These enzymes are commonly produced by bacilli and mycelial fungi

(Li et al., 2023) but not by lactic acid bacteria or yeasts (Gänzle and Follador, 2012). Phytate hydrolysis as well as the degradation of other anti-nutritive components including raffinose-family oligosaccharides and lectins is relevant in fermentation of pulses and cereals but not in milk (Gänzle, 2020; Tsuji et al., 2015). Pulses, as any other plant material, contain phenolic compounds and the ability of lactic acid bacteria to hydrolyse glycosides of secondary plant metabolites or to convert phenolic acids to flavor volatiles or other bioactives will impact product quality (Gaur and Gänzle, 2023; C. Li et al., 2021). Last but not least, milk fat is composed of diverse fatty acids including C4 to C7 short chain fatty acids that are odor-active upon release from the triglycerides (McSweeney et al., 2020) but short chain fatty acids are generally absent in plant oils. Conversely, unpasteurized plant substrates have lipoxygenase activity which oxidizes unsaturated fatty acids and generates the “beany” flavor of protein preparations from pulses. This flavor defect requires heterofermentative lactobacilli to reduce the odor-active aldehydes to alcohols with a much higher odor threshold (Sugahara et al., 2022).

The primary acidification cultures in cheese making and in fermentation of plant cheeses also differ. Cheese-making always involves primary acidification with a traditional back-slopped starter culture, or defined strain starter cultures derived from traditional cultures that contain *Streptococcus thermophilus* with *Lactobacillus delbrueckii* or *Lactobacillus helveticus*, or *Lactococcus lactis* and *Lactococcus cremoris* in association with *Leuconostoc* spp. (Parente et al., 2017). *L. delbrueckii* is highly specialized on lactose as substrate (van de Guchte et al., 2006) and unlikely to perform well in any plant fermentation. *Lc. lactis* and *Leuconostoc* spp. also occur in association with plants and in spontaneous plant fermentations and may be suitable for production of plant-based cheese analogues (Sooresh et al., 2023; Strafella et al., 2021). The traditional fermentation of soy (Tables 1 – 3) and other plant fermentations (Gänzle, 2022) relies on dozens of other species of lactic acid bacteria that may be more suitable for fermentation of plant-based cheese analogues than either lactococci with *Leuconostoc* spp. or the current default *Lp. plantarum* or *Lc. casei* (Mefleh et al., 2021). While the commercial availability of starter cultures dedicated to plant-based dairy and meat alternatives is increasing, these do not yet take advantage of the biodiversity of lactobacilli with potential use in plant-based cheese analogues. For example, the use of heterofermentative lactobacilli likely is a necessity to control the "beany" flavor caused by lipid oxidation products (Sugahara et al., 2022).

The succession of fermentation microbiota also differs in cheese ripening and traditional soy fermentations. Cheese making relies on acidification by lactic acid bacteria, followed by ripening with diverse ripening cultures. Depending on the type of cheese, microorganisms during ripening include non-starter lactic acid bacteria, propionibacteria, *P. roqueforti* or the surface cultures *Penicillum camemberti* in association with yeasts including *Debaryomyces hansenii* and *Geotrichum candidum*. Bacterial red smear surface cultures include *Brevibacterium* spp. and *Corynebacterium* spp. in addition to a large diversity of other microbes (Fox et al., 2017; Irlinger et al., 2017; Wolfe et al., 2014). In traditional Asian pulse fermentations, this sequence is reversed; hydrolytic cultures including mycelial fungi and / or bacilli grow first, followed by growth of yeasts and lactic acid bacteria to develop the texture, taste and odor of the product (Figures 1 and 4). Because most lactobacilli do not express extracellular protease activities (Zheng et al., 2015), a pre-fermentation with protease-producing microbes may be suitable for products that are composed of pasteurized ingredients.

The fermentation of cheeses and of traditional pulse fermentations is similar in one major aspect: Microbial metabolism converts sugars, proteins and lipids to taste-active compounds and odorants. The composition of ripening microbiota in cheeses and fermented soy food overlaps to some extend and includes e.g. *Lacticaseibacillus* spp. and *Lactiplantibacillus* spp.. Thus, the formation of tastants and odorants in cheeses and plant-based cheese-analogues may involve comparable microbes, metabolic pathways, and flavor-active compounds.

Carbohydrate metabolism is an important way to produce flavor compounds. In cheese making, the flavor compounds such as acetate, diacetyl and acetoin are generated through the metabolism of lactose and citrate by the starter culture (Lo et al., 2018; McSweeney et al., 2017). The conversion of citrate to diacetyl has also been described for plant-based fermentations with addition of lemon or apple juice as a source of citrate (Comasio et al., 2021).

Proteolysis and catabolism of amino acids are the major process for the flavor formation of cheese (Yvon and Rijnen, 2001). The molds and some of lactic acid bacteria in cheese aging possess comprehensive proteolytic systems, which can accelerate cheese ripening and convert the peptides that are produced by protease and peptidase originating from milk, coagulant and starter cultures to small peptides and amino acids (Griffiths and Tellez, 2013; McSweeney, 2017). Some of peptides and amino acids directly contribute to the flavor of cheese; the bitter peptides produced in aging process provided ripened Cheddar cheese with a perception of bitterness (Karametsi et al., 2014), and glutamic acid generated in ripening phase imparts umami taste (Ganesan and Weimer, 2017; Toelstede and Hofmann, 2008). Proteolysis is also a critical source for the production of flavor compounds in fermented soy foods; glutamic acid was as the most abundant of free amino acids in ripened *sufu* and several hydrophobic amino acids such as Phe, Leu, and Val contributed to bitterness of *sufu* (Xie et al., 2018).

In cheese ripening, yeasts and some lactic acid bacteria transform amino acids including valine, leucine, isoleucine, phenylalanine, tryptophan, tyrosine and methionine into odor volatiles such as aldehydes, alcohols and carboxylic acids by amino acid catabolism pathway/Ehrlich pathway

(Dzialo et al., 2017; Yvon and Rijnen, 2001). The sulphur compounds including methional, methanethiol, dimethyldisulphide and dimethyltrisulphide are also major aroma compounds of cheese, resulting from methionine degradation by cheese microorganisms such as *Brevibacterium lines* and *Geotrichum candidum* through amino acid elimination pathway (Berger et al., 1999; Dias and Weimer, 1998). However, the sulphur compounds are usually absent from fermented soy foods due to the limitation of methionine in the soy protein.

Free fatty acids are either aroma compounds such as butyric acid and hexanoic acid, or important precursors of aroma compounds such as esters, ketones, lactones, and secondary alcohols (Thierry et al., 2017). Cheese ripening microorganisms including *Propionibacterium freudenreichii*, *Yarrowia lipolytica*, *Geotrichum candidum*, and *Penicillium* spp. have high lipase activities, releasing free fatty acids from fat (Abeijón Mukdsi et al., 2014; Corzo and Revah, 1999; Gaborit et al., 2001; McSweeney and Sousa, 2000). During sufu ripening, a variety of ethyl esters were synthesized from the ethanol in dressing mixtures and fatty acids (Liang et al., 2019; Yao et al., 2021).

The salt content directly influences the overall acceptance of food. In order to inhibit the growth of undesirable microbiota, the high concentrations of salt solution are frequently utilized in the fermentation of *douchi* and *sufu*. The salt contents of final *douchi* and *sufu* products range from 5 to 18%, imparting a very salty taste to these two products. However, the salt content of cheese varies among different-type products, ranging from 0.5 to 6% (Guinee, 2004), which is significantly lower than that in *douchi* or *sufu*. There were more high salt tolerant microorganisms survived in post-fermentation of *douchi* or *sufu*, compared to those in cheese ripening.

Conclusions

Fermented soy foods exhibit the unique characteristics as a result of specific fermentation process and microbial activity. The heating step inactivates the vegetative microorganisms and enzymes in raw soybeans prior to the major fermentation. Thus, with the exception of stinky tofu, fermented soy foods use fungi and *Bacillus* spp. as starter cultures to produce a variety of hydrolytic enzymes to decompose proteins, polysaccharides and lipids at the initial stage of fermentation. There is no addition of salts in the fermentation of *natto* and *tempeh*. Their flavor and quality are highly related to the microbial activity of starter cultures. However, *douchi* and *sufu* undergo a long-time maturation and the addition of salts is essential to control the undesirable microorganism growth during the maturation. The lactic acid bacteria and yeasts dominate at the end of post-fermentation and finally impart the characteristic flavor and texture to the final products by a series of microbial enzymatic reactions. The understanding of major fermentation microbes and the major pathways for flavor formation in the production of traditionally fermented soy may provide helpful templates for the design of plant-based fermented products.

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Figure legends

Figure 1. Flowchart for the fermentation of *Mucor*-type and *Aspergillus*-type *Douchi*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The seasoning or dressing mixture contains 5 – 18% salt (Endo et al., 2014; He et al., 2016; Lite, 2005; Zhang et al., 2007) and additionally includes mixture of dry ground ginger, shallots or garlic (Zhang et al., 2007), a small amount of sugar, and selected spices such as capsicum paste (Lite, 2005), 5% fermented rice and 5% liquor (He et al., 2016), Chinese prickly ash, fresh hot pepper paste, and dry hot pepper powder

(Liu et al., 2012). The moisture content of the final product ranges from 35 to 50% (Lite, 2005); the final pH ranges from pH 6.5 to 6.9 (Wang et al., 2010a). The photo of douchi at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada.

Figure 2. Flowchart for fermentation of *natto*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The moisture content of the product is 59.5% (a_W 0.93 to 0.97)(Kim et al., 2012), the pH ranges from 4.9 to 7.5 (Kim et al., 2012; Tsai et al., 2007). The surface of *natto* is covered with slime or mucus that consists of 58 % of γ-polyglutamic acid and of 40% of polysaccharides (Saito et al., 1974). The photo of *natto* at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada.

Figure 3. Flowchart for the production of *tempeh*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The moisture content of the final product is about 58%, the pH ranges from 6.6 to 7.1 (Sparringa and Owens, 1999). The photo of *tempeh* at the lower left was available under a Creative Commons License on Aug 12, 2023 on https://en.wikipedia.org/wiki/Tempeh.

Figure 4. Flowchart for the production of mould-fermented and bacteria *sufu*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The final products have a moisture content of 58-73% (Han et al., 2001b, 2001a), a salt content of 6-15% (w/w)(Han et al., 2001a, 2004a), an ethanol content of 0-7% (v/v)(Han et al., 2004a) and a pH of 5.25-7.45 (Han et al., 2001a). The dressing

mixture contain one or more of the following: Red sufu, salt (10-14%), angkak (2%), alcoholic beverage (final alcohol content, 5-12% v/v), sugar (5-10%), soybean paste (3-5%), Chiang (wheat-based miso, 3%)[2,7]; the dressing mixture for white sufu is similar but contains no angkak (red rice fermented with *Monascus purpureus*); Grey sufu, soy whey, salt (14%). The photo of *sufu* at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada..

Figure 5. Flowchart for fermentation of stinky tofu (*choudoufu* or smelly tofu). Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. pH of stinky tofu brines: 5.20~7.72(Chao et al., 2008b), salt of stinky tofu brines: 1% (Chao et al., 2008b; Teng et al., 2004). The photo of stinky tofu at the lower left was available under a Creative Commons License on Aug 12, 2023 on https://en.wikipedia.org/wiki/Stinky_tofu.

Table 1. Fermentation microorganisms identified in *Aspergillus*-type- and in *Mucor*-type douchi.

Douchi		**Fermentation Microorganism**
Aspergillus-type *douchi*	Pre-	*Aspergillus oryzae, Bacillus subtilis, Aspergillus niger, Aspergillus egypticus*
	Post-	*Anaerosalibacter, Pseudomonas, Bacillus, Staphylococcus, Enterococcus, Lactococcus, Weissella, Pediococcus, Pichia, Aspergillus, Lichtheimia, Petromyces, Rhizopus, Penicillium, Candida, Saccharomyces*, and *Debaryomyces*
Mucor-type *douchi*	Pre-	*Mucor* spp., *Actinomucor elegans*
	Post-	*Tetragenococcus halophilus, Zygosaccharomyces rouxii, Candida versatilis, Staphylococcus, Bacillus, Aspergillus*, and *Penicillium*

With information from: (Chen et al., 2007, 2012, 2014; T. Chen et al., 2011a, 2011b; He et al., 2016; Li et al., 2018; Lite, 2005; Yang et al., 2019, 2016; Zhang et al., 2007b, 2021).

Table 2. Fermentation microorganisms identified in *tempe* or *tempeh*

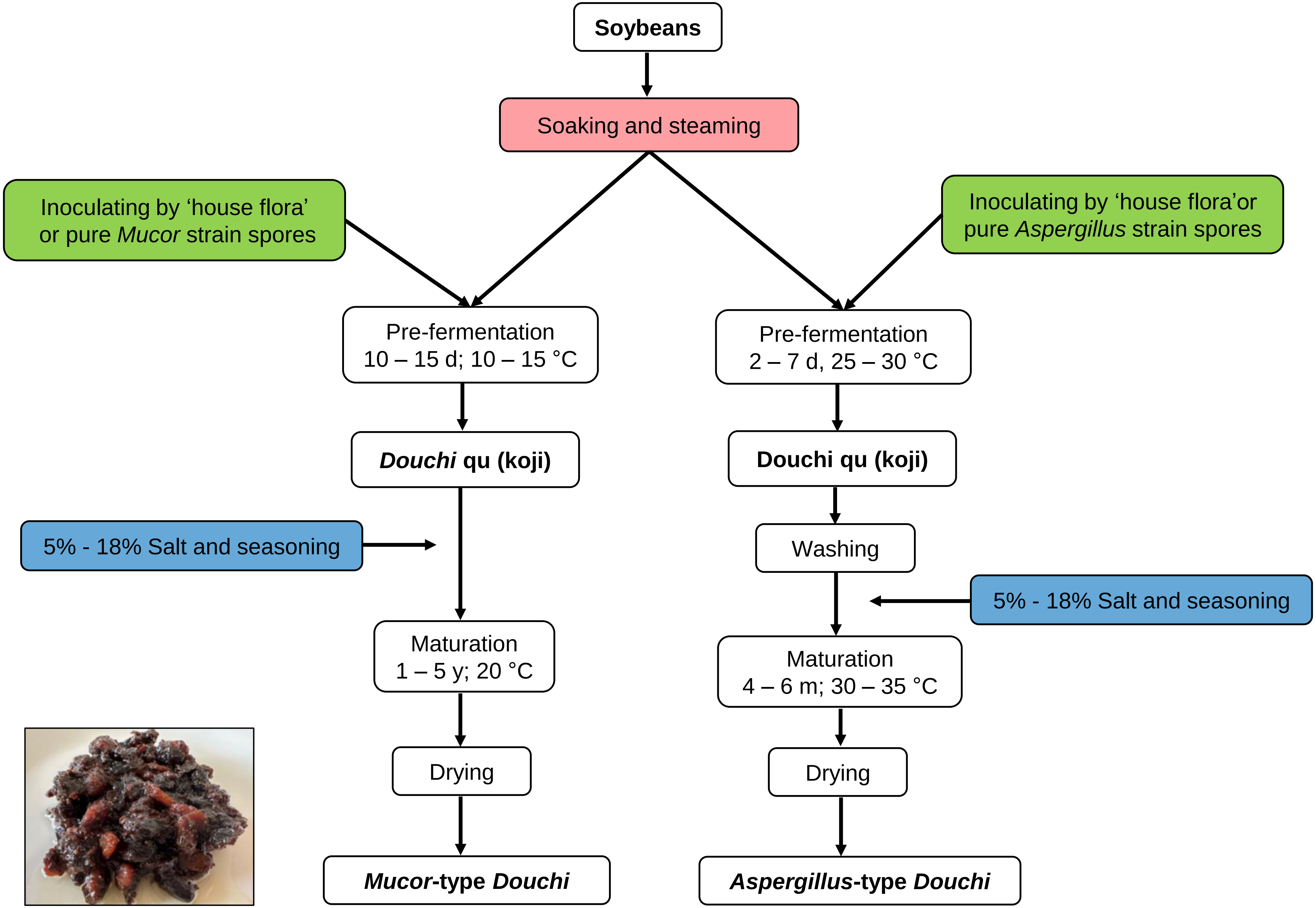
Tempeh		**Fermentation Microorganism**
Tempeh	Pre-	*Klebsiella pneumoniae, Klebsiella ozaenae, Enterobacter cloacae, Enterobacter agglomerans, Citrobacter diversus, Bacillus brevis, Pichia burtonii, Candida diddensiae, Rhodotorula rubra, Lacticaseibacillus casei, Enterococcus faecium, Staphylococcus epidermidis,* and *Streptococcus dysgalactiae,* and co-inoculant *Lactiplantibacillus plantarum*
	Post-	*Rhizopus* spp. (*R. arrhizus, R. delemar*, *R. microsporus* and *R. stolonifer*), *Bacillus pumilus, Bacillus brevis, Streptococcus faecium, Lacticaseibacillus casei, KIebsiella pneumoniae Enterobacter cloacae, Micrococcus varians, Trichosporon beigelii, Clavispora (Candida) lusitaniae, Candida maltose, Candida intermedia,Yarrowia lipolytica, Mucor indicus and Mucor circinelloides* , and/or co-inoculants: lactobacilli, *Citrobacter freundii, Klebsiella pneumoniae,* and *Propionibacterium freudenreichii*

With information from (Mogessie Ashenafi and Busse, 1991; Feng et al., 2005; Hartanti et al., 2015; Keuth et al., 1994; Mulyowidarso et al., 1990, 1989; Nout et al., 1987a; Samson et al., 1987; Signorini et al., 2018; Sjamsuridzal et al., 2021; Wiesel et al., 1997)

Table 3. Fermentation microorganisms identified in fermentation of *sufu*

Type of product		**Fermentation Microorganism**
Mould-fermented sufu	Pre-	*Actinomucor* spp., *Mucor spp.* and *Rhizopus spp. (Actinomucor elegans, Actinomucor taiwanensis, Actinomucor repens, Mucor racemosus, Mucor Wutongqiao, Mucor circinelloides, Mocur hiemalis, Mucor flavus, Rhizopus microsporus* var. *microspores,* and *Rhizopus oryzae)*
	Post-	**Pure culture fermentation:** *Enterobacter, Acinetobacter, Lactococcus, Tetragonococcus, Bacillus, Enterococcus, Streptococcus, Macrococcus, Monascus and Aspergillus;* **Spontaneous fermentation:** *Bacillus, Enterococcus, Lactococcus, Leuconostoc, Lactobacilli, Weissella, Enterobacter, Tetragenococcus, Trabulsiella, Sphingobacterium, and Stenotrophomonas, Sterigmatomyces, Debaryomyces, Tausonia, Pichia, Candida, Geotrichum, Fusarium, and Actinomucor*
Bacteria-fermented sufu	Pre-	*Bacillus* spp. or *Micrococcus spp.*
	Post-	**Spontaneous fermentation:** *Enterococcus avium, Enterococcus faecalis, Staphylococcus carnosus, Leuconostoc mesenteroides, Staphylococcus saprophyticus, Streptococcus lutetiensis, Kocuria rosea, Kocuria kristinae, Bacillus pumilus, Bacillus cereus group*[*], and *Bacillus subtilis*

[*]The organism was identified by sequencing of 16 rRNA genes, which does not differentiate between *B. cereus*, *B. thuringiensis*, *B. anthracis* and closely related species. With information from (Feng et al., 2013; Han et al., 2001b; He and Chung, 2020; Huang et al., 2018; Li et al., 2022; X. Li et al., 2021; Liang et al., 2019; Tan et al., 2020; Wan et al., 2020; Xu et al., 2020).



Soybeans

Soaking and steaming

Inoculating by ‘house flora’
or pure *Mucor* strain spores

Inoculating by ‘house flora’or
pure *Aspergillus* strain spores

Pre-fermentation
10 – 15 d; 10 – 15 °C

Pre-fermentation
2 – 7 d, 25 – 30 °C

***Douchi* qu (koji)**

Douchi qu (koji)

5% - 18% Salt and seasoning

Washing

5% - 18% Salt and seasoning

Maturation
1 – 5 y; 20 °C

Maturation
4 – 6 m; 30 – 35 °C

Drying

Drying

Mucor*-type *Douchi

Aspergillus*-type *Douchi

Soybeans

Soaking and steaming

Inoculating by pure *Bacillus subtilis* (natto) strain spores

Packing

Fermentation
16 – 20 h; 40 – 42 °C

Maturation
24 h; 4 – 10 °C

Natto

Raw beans, legume, or grain

Spontaneous fermentation or inoculating by pure *Lactiplantibacillus plantarum* strains

Soaking (and acidification)

Dehulling and washing

Organic acid (optional)

Boiling

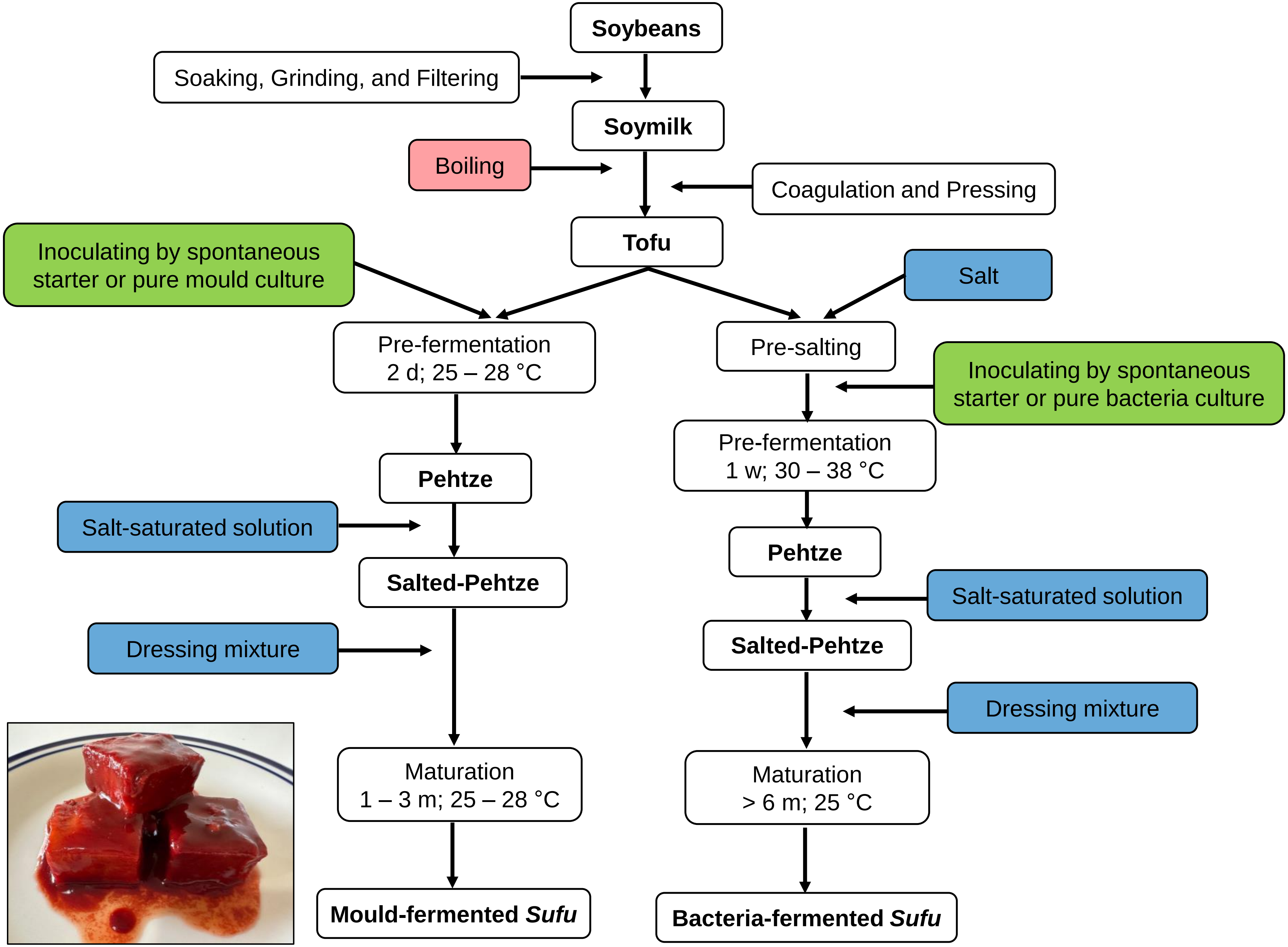
Cooling

Inoculating by pure *Rhizopus* strain spores

Packing

Fermentation
24 – 48 h; 24 – 30 °C

Tempeh



Soybeans

Soaking, Grinding, and Filtering

Soymilk

Boiling

Coagulation and Pressing

Tofu

Inoculating by spontaneous starter or pure mould culture

Pre-fermentation
2 d; 25 – 28 °C

Pehtze

Salt-saturated solution

Salted-Pehtze

Dressing mixture

Maturation
1 – 3 m; 25 – 28 °C

Mould-fermented *Sufu*

Salt

Pre-salting

Inoculating by spontaneous starter or pure bacteria culture

Pre-fermentation
1 w; 30 – 38 °C

Pehtze

Salt-saturated solution

Salted-Pehtze

Dressing mixture

Maturation
> 6 m; 25 °C

Bacteria-fermented *Sufu*

Vegetables, mashed tofu and salt with or without meats

Natural fermentation or Inoculating by original stinky tofu brine

Fermentation
0.5 – 2 y; 20 – 30 °C

Stinky tofu brine

Soybeans

Soaking, Grinding, and Filtering

Soymilk

Boiling

Coagulation and Pressing

Tofu

Soaking
1 – 6 h; 27 – 30°C
(20 h; 5°C)

Drip-drying and frying

Stinky tofu