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Quality improvement of soymilk as influenced by anaerobic grinding method and calcium addition

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ABSTRACT

Soymilk is one of the plant-based milk products containing great nutritional content and has an unpleasant beany flavour and low calcium content. In this study, different grinding methods and calcium salts fortification were applied to improve the flavour, calcium content, and stability of soymilk upon calcium addition. According to the results, preparing soymilk using the anaerobic grinding method significantly reduced the lipid oxidation (0.4781 meq peroxide/kg) and increased the sulfhydryl group content (24.25 μmol/g), compared to preparing soymilk using the conventional grinding method (0.7668 meq peroxide/kg and 15.68 μmol/g). As the results of different grinding methods, oxygen-free soymilk (OFS) has a lower amount of volatile compounds (75.04 μg/mL) than regular soymilk (RS; 966.65 μg/mL). The addition of calcium salts increased the particle diameter and viscosity of soymilk, where the significant increase was shown in calcium-enriched soymilk prepared using conventional grinding. The addition of calcium lactate reduced soymilk pH while calcium carbonate addition showed the opposite results. Soymilks enriched with calcium lactate have equivalent calcium bioavailability (28.99 and 31.21%) with cow's milk (29.47%) and significantly higher than soymilks enriched with calcium carbonate (23.73 and 22.35%). Thus, this study suggests the preparation of soymilk using the anaerobic grinding method with the addition calcium lactate as the calcium source. This combination can produce calcium-enriched soymilk with high acceptability without altering the initial sensory properties.

1. Introduction

In the last few decades, plant-based milk products are widely sold globally and attract consumers' attention; one of the most favourite plant-based milk products is soymilk. It is generally known as milk substitutes since it has high-quality protein and contains considerable amounts of polyunsaturated fatty acids and essential amino acids except for methionine (Iwuoha & Umunnakwe, 1997; Rivas et al., 2002; Zhou et al., 2019). Soymilk also has lower lactose and cholesterol content than cow's milk, making it suitable for lactose intolerance people or those allergic to cow's milk (Bricarello et al., 2004; Hajirostamloo, 2009; Sethi et al., 2016). Some researchers have previously reported that soymilk may improve human immunity and has anti-cancer effects due to its phenolic content (Lai et al., 2013; Wahle et al., 2010). Additionally, it also contains a substantial amount of isoflavones (genistein, daidzein, and glycetein) (Rizzo & Baroni, 2018) and some phytochemicals such as saponins, sterols, and phytic acid, which may increase its potential health benefit (Kang et al., 2010).

Despite the abundant benefits, soymilk has been reported with its unpleasant beany flavour, which affects the enjoyment of consuming this beverage, especially for teenagers and Western consumers (Min et al., 2005). The unpleasant beany flavour was caused by the oxidation of polyunsaturated fatty acid to other compounds (aldehydes, alcohols, ketone, and furan) in the presence of oxygen and lipoxygenase (LOX) as the catalyst (Ma et al., 2015; Navicha et al., 2018). Furthermore, soymilk also has another drawback due to its relatively low calcium content (0.25 mg/g) when compared to cow's milk (1.2 mg/g) and human breast milk (0.32 mg/g) (Prabharaksa et al., 1989; USDA, 2015). Calcium is an

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essential mineral for bone development, teeth, muscles, nerves, and enzyme function in the human body (Gerstner, 2003). Inadequate calcium intake can lead to health problems, such as muscle contraction, neurotransmitter secretion, blood coagulation, and osteoporosis in later life (Pathomrungsiyounggul et al., 2013; Theobald, 2005).

The unpleasant beany flavour can be controlled by modifying the processing method because the conventional method establishes conditions for oxidation to occur during soaking and grinding of soybean (Donkor et al., 2007). Grinding soybean under anaerobic conditions could be another option to reduce the beany flavour and limit oxidation in soymilk. Simultaneously, calcium content in soymilk can be increased to be equivalent to cow's milk by fortifying calcium salts. Some calcium salts have been approved and considered safe for calcium fortification by U.S. Food and Drug Administration, such as calcium carbonate, calcium chloride, calcium lactate, calcium citrate, calcium gluconate, and many more (Fairweather-Tait & Teucher, 2002). However, in this study, only calcium lactate and calcium carbonate were used as the calcium source; since the investigation about these calcium salts as the calcium source in soymilk is still limited.

Based on our knowledge, there is no study combining the anaerobic grinding method with calcium addition to improve soymilk qualities. Based on our preliminary experiment, this combination affected soymilk properties and showed the potential to produce stable calcium-enriched soymilk with a lighter flavour. The stability of calcium-enriched soymilk is the major factor that should be controlled since visible coagulation occurs when calcium is added to soymilk (Kaharso et al., 2020; Pathomrungsiyounggul et al., 2010). Therefore, this study aims to improve soymilk quality by using different grinding methods and calcium salts fortification. The stability and sensory evaluation of calcium-enriched soymilk prepared from different grinding methods were also investigated. It is expected that this research can produce stable calcium-enriched soymilk with low unpleasant beany flavour.

2. Materials and methods

2.1. Materials

Soybeans (*Glycine max*) Heinong 54 were purchased in 2020 from a local market (Wuxi, China) and stored at 4 °C until used. Calcium salts used in this study were calcium lactate (ca-lactate; food-grade, Wangwang Biological Technology Co., Ltd., China) and calcium carbonate (ca-carbonate; food-grade, Shengda Biological Technology Co., Ltd., China). Other chemicals used were purchased from Sinopharm Chemical Reagents Co., Ltd., China. All water used for calcium analysis were prepared using Milli-Q water (Millipore Corporation, USA).

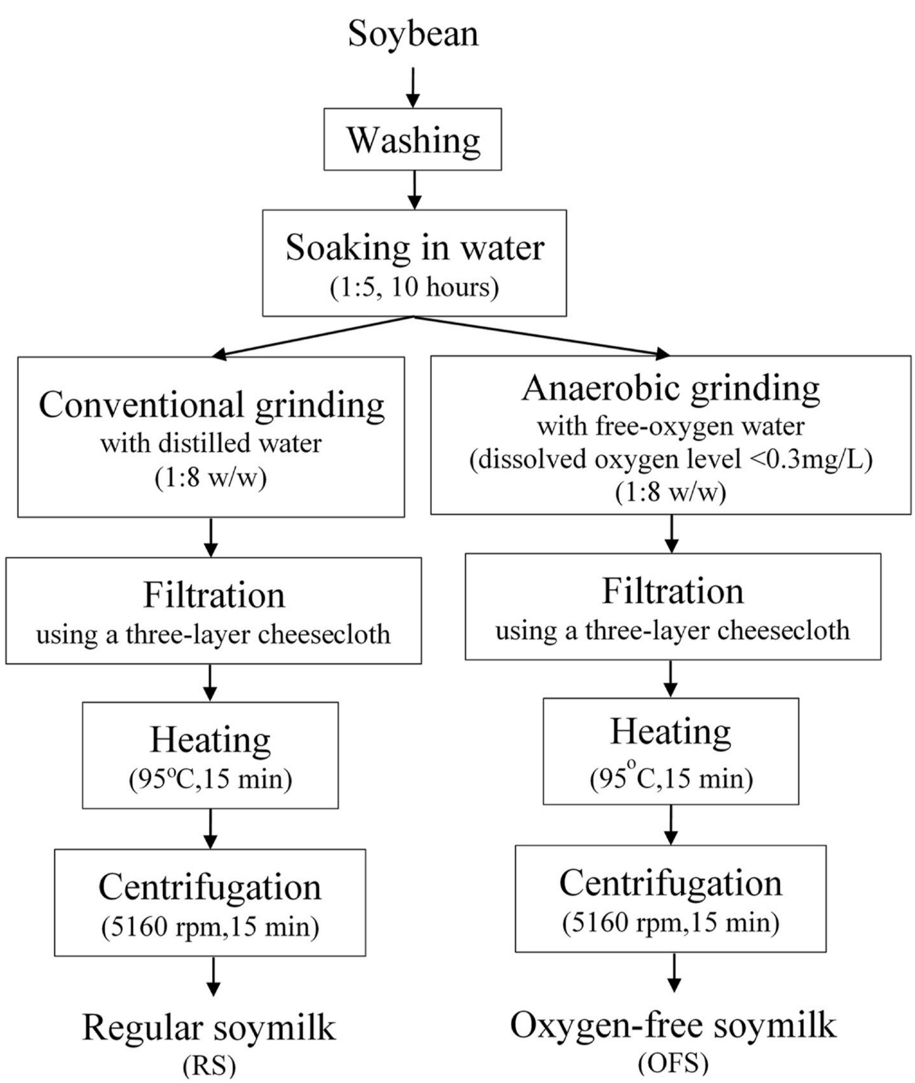

Fig. 1. Methods of soymilk preparation applied in this study.

2.2. Preparation of soymilk

Soymilk from different grinding methods was prepared according to the procedure in Fig. 1. Soybeans were washed three times using tap water then deionized water before soaked in soybean: water ratio of 1:5 (w/w) for 10 h at room temperature in a dark room. The swollen soybeans were sieved, rinsed with distilled water, and ground using a soymilk maker (JYL-C012, Joyoung, Hangzhou, China) at room temperature with two different grinding methods (conventional and anaerobic). For the conventional grinding method, soybeans were ground with distilled water in soybean: water ratio of 1:8. Meanwhile, for the anaerobic grinding method, the oxygen-free water was added in the same ratio as the conventional method. The oxygen in oxygen-free water was removed by vacuum deaeration using a water ring vacuum pump (3.3 kPa, 2880 rpm/min; Shanghai Feilu Pump Technology Co., Ltd.) to reach dissolved oxygen level <0.3 mg/L. The slurries from both grinding methods were filtered through a three-layer cheesecloth; then heated at 95 °C for 15 min and cooled to room temperature using an ice bath for about 20 min. Next, heated soymilks were centrifuged at 5160 rpm for 15 min with a high-speed refrigerated centrifuge (Hitachi Himac CR21GII, Japan) to remove the remaining soybean residue. The supernatants from both grinding methods are now referred to as regular soymilk (RS) and oxygen-free soymilk (OFS).

2.3. Soymilk oxidation analysis

2.3.1. Peroxide value

Peroxide value (PV) as the primary oxidation products were used to indicate the lipid oxidation in RS and OFS. Lipid from both samples was extracted using chloroform-methanol extraction and evaporated to dryness under nitrogen. A colorimetric ferric-thiocyanate method (Shantha & Decker, 1994) was used to analyze PV, which was performed on the same day of lipid extraction. The results were expressed as meq peroxides/kg lipid.

2.3.2. Sulfhydryl group (SH) content

Soymilks were treated with anhydrous ethanol to precipitate the protein from the emulsion system (Ou et al., 2004). The mixture of 2 mL soymilk and 18 mL ethanol were centrifuged at 3000 rpm for 15 min; then, the precipitate was re-suspended twice in 10 mL of ethanol. Later, the ethanol was evaporated from the tubes under nitrogen. The precipitates were then dispersed in 0.1 M sodium phosphate buffer (PBS buffer, pH 7.0) and centrifuged again at 4000 rpm for 10 min. The supernatants were then separated and used to analyze the SH content using 4,4′-dithiodipyridine method as described in Ruan et al. (2013). The protein concentration was determined using the micro-Kjeldahl method (AOAC, 2005).

2.4. Identification of volatile compounds

Volatile compounds were determined according to the method of Zhang et al. (2020) with minor modification. A gas chromatography-mass spectrophotometry (GC-MS) system (QP 460, Bruker, Germany) equipped with a DB-Wax column (0.25 μm, 30 m × 0.25 mm) was used. The sample in the vial was incubated (50 °C, 10 min) and adsorbed the volatile compounds. The adsorbed volatiles compounds were then desorbed in the injector port without splitting (260 °C, 7 min). The column temperature program was set as follows: the initial temperature was kept at 40 °C for 3 min, then increased to 100 °C at 6 °C/min, and finally increased to 230 °C at 10 °C/min and held for 7 min. The MS detector was operated in electron-impact mode (ionization energy of 70 eV) with a scan range from 40 to 350 m/z, and the temperature of MS source was set at 200 °C. The data was analysed using the National Institute of Standards and Technology database and Wiley 6.0 library software.

2.5. Preparations of calcium-enriched soymilk

Calcium-enriched soymilks were prepared based on our previous research (Kaharso et al., 2020). Firstly, RS and OFS were diluted with deionized water to equalize the total solid of both sample to 7% (w/v); then heated to 50 °C using a heated magnetic stirrer (Pioway Medical 79–1, China) before adding tripotassium citrate as chelating agents to stabilize soymilk upon calcium addition. Ca-lactate and ca-carbonate were then added to reach the equivalent calcium content in cow's milk (1.2 mg/ml). Later, the calcium-enriched samples were pasteurized in sterilized glass bottles at 95 °C for 15 min and cooled to room temperature in an ice bath for about 20 min, before stored under refrigeration (4 °C).

2.6. Calcium dissolution rate

The dissolution rates of calcium salts in water and soymilk were determined based on the method by Chaiwanon et al. (2000) and Dechapinan et al. (2017) with modification. Briefly, 1% (w/v) of each calcium salt was added to water and soymilk, then stirred for 360 min. During stirring process, samples were collected under different interval times (0.5, 1.5, 5, 15, 60, 180, and 360 min). Later, each sample was centrifuged at 3500 rpm, 20 °C, for 15 min to remove the insoluble components. The calcium content in the supernatant was then analysed using Atomic Absorption Spectrometry (AA 240 FS, Agilent, Australia) and the calcium dissolution rate was calculated by using the following equation: Calcium dissolution rate (%) = (A-B)/C x 100; where A = calcium content (mg/g) in the supernatant, B = calcium content (mg/g) of water or soymilk, and C = total calcium (mg/g) in salts.

2.7. Soymilk pH

A digital pH meter (FiveEasy Plus FE28, Mettler Toledo, China) was used to measure the pH of the sample at 25 °C, and results were expressed as pH units.

2.8. Effective particle diameter

The effective particle diameter was determined according to Kaharso et al. (2020). Each sample was diluted 100 times before measured at 25 °C with dynamic light scattering (Nano Brook Omni, Brookhaven Instrument, USA). Protein was selected as materials (1.450 refraction index), while water was used as the dispersant.

2.9. Rheological properties

Rheological properties of soymilks were determined using a controlled-stress rheometer (MCR 301, Anton Paar Tru Gap TM Instrument, Austria) equipped with parallel plate geometry (25 mm diameter, 1 mm gap). The shear stress of each sample was measured at 25 °C, with shear rates from 0.1 to 300/s. The model used to fit the flow behaviour data was Power-law fluid according to the formula described by Navicha et al. (2018).

2.10. Confocal laser scanning microscopy (CLSM)

The microstructures of samples were visualized using Zeiss LSM 880 (Carl Zeiss Jena, Germany). Protein and oil were stained by fluorescein isothiocyanate (FITC) and Nile red (Sigma-Aldrich, China), respectively. Briefly, 20 μL 0.05% (w/v) FITC solution and 20 μL 0.05% (w/v) Nile red solution were added to 1 mL sample, then mix evenly. Sample was then incubated for 20 min in a covered box to avoid light exposure. Later, 10 μL of sample was added to the glass slide, covered with a cover glass, and sealed with nail polish. FITC was analysed at wavelength of 518 nm and Nile red at 488 nm.

2.11. In vitro calcium digestion

A method by Cámara et al. (2005) with minor modifications was used to estimate the dialyzable calcium. Pasteurized cow's milk (Yili Industrial Group Co., Ltd, China) was used as a control for calcium-enriched soymilk samples. The pH of the 100 mL of the sample was adjusted to 2.0 with 6 N HCl before 1 mL of 2% (w/v) pepsin (Sigma-Aldrich, China) in 0.1 M HCl was added. The aliquot was incubated at 37 °C for 2 h using a shaking water bath. A cellulose dialysis bag (cut-off 12 kDa; Sinopharm Chemical Reagents Co., Ltd., China) containing 25 mL water and an amount of $NaHCO_3$ (equivalent to the titratable acidity of the combined pepsin digest pancreatin-bile extract mixture) was placed in a beaker containing 50 mL of the pepsin digest aliquot. The titratable acidity was defined as the number of equivalents NaOH required to titrate the combined mixture of pepsin digest pancreatin-bile salts to pH 7.5. Incubation was continued for 30 min before a mixture consisting of 4% pancreatin and 6% (w/v) bile salts (Sigma-Aldrich) in 0.1 M $NaHCO_3$ was added and incubated again for 2 h. Then, the dialysis bag was removed and cleaned with Millie-Q water. The total calcium content in sample and calcium content in dialysis bag was measured using Atomic Absorption Spectrometry. The calcium bioavailability was calculated as follows: Calcium bioavailability (%) = D/T x 100; where D = dialyzable calcium content (mg/g) and T = total calcium content in sample (mg/g).

2.12. Sensory evaluation

The sensory properties were evaluated by 15 panellists from Jiangnan University, China. The panellists consisted of 8 males and 6 females with ages ranging from 22 to 36 years. All soymilks and calcium-enriched soymilks were dispensed in plastic cups and coded with three-digit random numbers. The evaluation data were recorded and calculated mean scores of each attribute compared with samples. The attributes tested were mouthfeel, beany flavour, bitterness, and overall acceptance. The 9-hedonic scales (Lawless & Heymann, 2010) were used to describe the panellists' preferences.

2.13. Statistical analysis

All experiments were carried out at least twice in triplicate. Data shown are represented as means ± standard deviations and analysed using SPSS for Windows (version 19.0, SPSS Inc., IL). The significant difference was calculated at $P < 0.05$ using Duncan Multiple Range Test.

3. Result and discussion

3.1. Effect of different grinding method on soymilk properties

3.1.1. Oxidative degradation

Lipid oxidation of RS and OFS samples was assessed by monitoring lipid peroxide formation through peroxide value as represented in Fig. 2. Findings have previously reported that the PV of RS sample (0.7668 meq peroxide/kg lipid) was significantly higher ($P < 0.05$) than the OFS sample (0.4781 meq peroxide/kg lipid). The high value in PV is due to the oxygen present during the grinding process. Mora et al. (2018) stated that mechanical actions during grinding lead to cell disruption and oxygen inclusion, resulting in increased contact with reactive oxygen species. In other related studies on lipids oxidation, findings of Johnson and Decker (2015), O'Brien and O'Connor (2011), and Zhu and Sevilla (1990) revealed that oxygen in the form of triplet oxygen ($^3O_2$) reacts with the alkyl radical (unsaturated fatty acid-free radical) to form a covalent bond and produce peroxy radicals that able to separate another hydrogen from other unsaturated fatty acids to create corresponding hydroperoxides new alkyl radical. LOX present in soymilk also affected the exposed lipids to produce hydroperoxides (Zhao et al., 2016); since LOX is the enzyme that catalyzes the oxidation of polyunsaturated lipids by molecular oxygen (Axelrod et al., 1981).

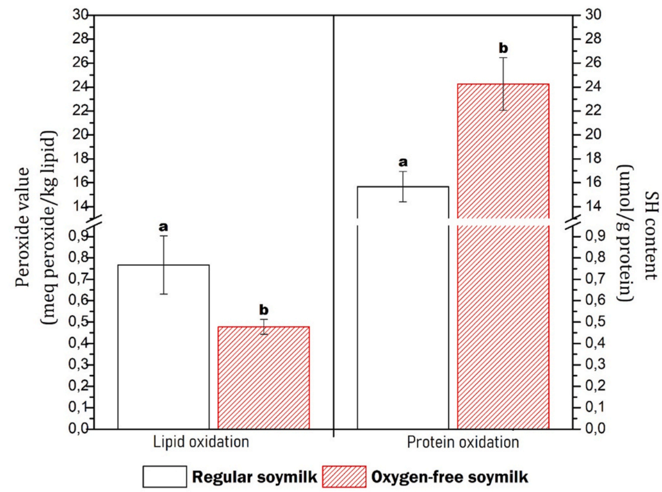

Fig. 2. Oxidative degradation of soymilk prepared by different grinding methods.

The SH content was conducted to evaluate the protein oxidation in soymilk prepared from different grinding methods. The reduction of SH content generally signifies that proteins were denatured, aggregated (through intermolecular disulfide bonds), or oxidized (Huang et al., 2006). Ye et al. (2015) reported that protein oxidation in peanut-based milk has negative impacts and reduces emulsion stability and altered particle size enlargement (Ye et al., 2013). Furthermore, higher oxidation could also promote the formation of insoluble components and cleavage of protein bonds (Davies, 2005). Fig. 2 shows that the SH content of RS sample was 15.68 μmol/g protein, significantly lower ($P < 0.05$) than OFS sample with 24.25 μmol/g protein. As reported in Obata et al. (1996), grinding soymilk under anaerobic conditions could limit the degradation of SH content which was closely related to the oxidative reaction by LOX. The lipid peroxide content was also the cause of this degradation since lipid peroxides could react with protein thiols and formed sulfonic acid as monomeric oxidation products (Little & O'Brien, 1967). In addition, the alteration of the protein structure by oxidative degradation can alter its binding ability with volatile compound and affects aroma as the sensory attributes (Mora et al., 2018). Even though oxidation in food generally focuses on lipid oxidation, protein oxidation must also be considered to produce great quality products.

3.1.2. Volatile compounds

The volatile compounds of soymilk prepared by different grinding methods are presented in Table 1. According to our results, the total volatile compound of OFS sample (75.04 μg/mL) was significantly lower ($P < 0.05$) than RS sample (966.65 μg/mL). It was observed that grinding soybean under anaerobic conditions efficiently reduced soymilk off-flavour. Among volatile compounds detected by GC-MS analysis, hexanal (308.48 μg/mL) has the highest amount in RS sample; meanwhile, in the OFS sample, 1-octene-3-ol (32.08 μg/mL) was the highest. Similar results were also shown in Lv et al. (2011), where hexanal was proved as the most pungent flavour compounds in traditional soymilk with 625 flavour dilution (FD) value, while 1-octene-3-ol was the second most pungent compounds (125 FD value). Both hexanal and 1-octene-3-ol, were reported to contribute to the unpleasant beany flavour in soybean products, besides other compounds such as 2-hexenal, 2-pentylfuran, 1-pentanol, 2-heptenal, 1-hexanol, 2,4-heptadienal, benzaldehyde, 2,4-decadienal (Lv et al., 2011; Navicha et al., 2018; Yuan & Chang, 2007; Zhou et al., 2019).

Based on our GC-MS results presented in Table 1, the beany flavour in soymilk was successfully reduced using a different grinding atmosphere. Similarly, the non-beany flavour compounds of OFS sample

Table 1
Quantitative comparison of volatile compounds in soymilk prepared from different grinding methods.

Volatile compounds	Relative quantities (μg/mL)		Odor description
	RS	OFS	
Pentanal	3.95 ± 0.83[a]	0.51 ± 0.11[b]	Grass, green
Hexanal	308.48 ± 51.54[a]	20.02 ± 8.03[b]	Cut grass, green
Trans-2-pentenal	3.72 ± 0.11	ND	Fatty
Heptanal	9.28 ± 0.21[a]	1.10 ± 0.28[b]	Rancid
2-hexenal	16.47 ± 2.86[a]	5.79 ± 6.06[b]	Leaf
2-pentylfuran	50.25 ± 40.94[a]	2.20 ± 0.91[b]	Green bean
1-pentanol	11.74 ± 1.23[a]	0.67 ± 0.61[b]	Alcoholic, wine
Octanal	6.17 ± 0.81[a]	0.60 ± 0.15[b]	Lemon, fruity
Trans-2-heptenal	29.25 ± 3.34[a]	0.87 ± 0.17[b]	Fruity, citrus
1-hexanol	263.05 ± 49.86[a]	4.37 ± 1.40[b]	Lemon, grass, green
Nonanal	32.79 ± 13.11[a]	2.74 ± 0.30[b]	Floral, citrus
2-octenal	33.56 ± 7.00[a]	0.61 ± 0.04[b]	Cucumber, vegetable
1-octene-3-ol	165.72 ± 31.01[a]	32.08 ± 5.56[b]	Mushroom,
Trans, *trans*-2,4-heptadienal	2.34 ± 0.85[a]	0.31 ± 0.08[b]	Fried, fatty
Decanal	1.48 ± 0.42[a]	0.72 ± 0.47[b]	Earthy, mushroom
Benzaldehyde	6.44 ± 2.43[a]	1.57 ± 0.82[b]	Almond
Trans-2-decenal	5.82 ± 2.14[a]	0.58 ± 0.60[b]	Orange, green
Trans, *trans*-2,4-decadienal	16.14 ± 2.22[a]	0.30 ± 0.06[b]	Fatty
Total compounds	966.65 ± 210.93[a]	75.04 ± 25.64[b]	

Relative quantities were calculated using the internal standard 2-methyl-3-heptanone. Means ± standard deviation in the same row with different letter are significantly different ($P < 0.05$).
ND; not detected.
Odor description (Lv et al., 2011; Zhang et al., 2020).

(octanal, nonanal, 2-octenal, and decanal) were also found lower than RS sample. In our study, the reduction of beany flavour was associated with the inactivation of LOX to catalyse the enzymatic reaction to generate beany flavour as the off-flavour in soymilk; since oxygen, as one of the reactant was kept out (Giri & Mangaraj, 2012). Researchers used blanching (Lv et al., 2011), hot temperature grinding (Lv et al., 2011; Mizutani & Hashimoto, 2004), and roasting (Navicha et al., 2018) to reduce beany flavour in soymilk. However, the utilization of high temperature during the process, increases the protein oxidation and affects the structure of protein in soymilk (Obata et al., 1996; Obata & Matsuura, 1993). Grinding soybean under anaerobic method proved to be an efficient way to produce soymilk with a lighter flavour and good properties.

3.2. Calcium dissolution rate

In our study, the dissolution rate was determined to express solubility rates of both ca-lactate and ca-carbonate in the water and soymilk medium. Gerstner (2003) stated that the solubility of calcium salts is one of the major characteristics of calcium fortification in beverages. The results of calcium dissolution rate are present in Table 2. As expected, the dissolution rate of both calcium salts in water and soymilk mediums was significantly increased ($P < 0.05$). The dissolution rate of calcium positively correlated with the stirring time. The dissolution rate of ca-lactate in water reached 94.81% at minute 1.5 and remained the same with no significant ($P > 0.05$) increased after that, while the dissolution rate of ca-carbonate continued increasing by reaching 53.44% at min 360. Meanwhile, the dissolution rate of ca-lactate significantly decreased ($P < 0.05$) in the soymilk medium, indicated by the calcium dissolution rate reaching 94.22% at 60 min of stirring. The same results were also shown on the dissolution rate of ca-carbonate in soymilk medium, where it only reached 36.71% when stirred for 360 min.

Table 2
The dissolution rate of calcium salts in water and soymilk medium.

Stirring time (minutes)	Water medium (%)		Soymilk medium (%)	
	Ca-carbonate	Ca-lactate	Ca-carbonate	Ca-lactate
0.5	2.70 ± 0.83[aA]	75.70 ± 2.58[aC]	0.99 ± 0.86[aA]	52.70 ± 1.36[aB]
1.5	3.22 ± 2.38[aA]	94.81 ± 1.89[bC]	2.61 ± 1.31[aA]	62.21 ± 2.25[bB]
5	3.27 ± 1.06[aA]	95.31 ± 1.75[bC]	3.01 ± 1.90[aA]	68.74 ± 1.59[cB]
15	6.41 ± 1.32[bA]	94.97 ± 1.88[bC]	7.13 ± 1.66[bA]	90.27 ± 1.52[dB]
60	14.76 ± 2.25[cB]	93.73 ± 2.33[bC]	10.95 ± 1.69[cA]	94.22 ± 1.30[eC]
180	46.88 ± 1.39[dB]	95.49 ± 2.64[bC]	16.53 ± 1.14[dA]	93.69 ± 2.04[eC]
360	53.44 ± 0.68[eB]	95.95 ± 2.46[bC]	36.71 ± 1.91[eA]	94.37 ± 1.03[eC]

a Means ± standard deviation in the same column with different lowercase letter are significantly different ($P < 0.05$).
A Means ± standard deviation in the same row with different capital letter are significantly different ($P < 0.05$).

From these results, it was observed that ca-lactate has a higher dissolution rate than ca-carbonate. Gerstner (2003) and Trailokya et al. (2017) reported that ca-lactate is one of the calcium salts with excellent solubility, making it favorable for fortification in beverages. On the contrary, ca-carbonate has lower solubility; the solubility could increase at lower pH (Goss et al., 2007), indicating that it is more suitable for low-pH food and beverages. Furthermore, the dissolution rate of calcium salts decreased when dispersed in the soymilk medium than water. The possible explanation for this was due to the presence of nutritional components such as carbohydrate, protein, and fat, which could affect the solubility of calcium salts in food. The experimental study of calcium dissolution rate in water and soymilk medium could be a better guidance for further application of calcium fortification in food and beverages.

3.3. Effect of different grinding method and calcium salt on soymilk stability

3.3.1. pH

Based on the results in Table 3, the pH of RS (6.62) has no significant difference ($P > 0.05$) with OFS (6.69). These findings indicated that different grinding methods did not influence soymilk pH. However, when ca-lactate was added to RS and OFS, the pH significantly decreased ($P < 0.05$) to 6.46 and 6.49, respectively. Similar results were also reported by Pathomrungsiyounggul et al. (2010), where addition of ca-lactate reduced soymilk pH. The reduction of soymilk pH was caused by the liberation of hydrogen ions due to its competition with calcium ions in the same binding sites of soy protein molecules (Kroll, 1984) and the phosphate group in soymilk phytate (Ono et al., 1993). On the other hand, the addition of ca-carbonate significantly increased ($P < 0.05$) the pH of RS and OFS to 7.23 and 7.28, respectively. The formation of CO_2 and H_2O caused the increase in soymilk pH due to the interaction between liberated hydrogen ions and carbonate portions (CO_3^-) of ca-carbonate (Comuzzo & Battistutta, 2018).

According to our results, it can be concluded that the calcium ions activity caused the changes in pH. Pathomrungsiyounggul et al. (2007) stated that pH is inversely correlated with calcium ion activity, where calcium ion activity increase as the pH decrease. Simultaneously, the solubility of calcium salts also affects the calcium ions activity because the more soluble the salts the more calcium ions in the solution available for reaction (Gerstner, 2003) and show greater effect in pH (Pathomrungsiyounggul et al., 2010). Therefore, it is crucial to control and maintain the pH of calcium-enriched soymilk around its initial pH; since the amount of calcium-protein bound rises rapidly when the pH was

Table 3
pH, effective particle diameter, N value, and K value of soymilk and calcium-enriched soymilk.

	pH	Eff. particle diameter (nm)	n value	K value (Pa.s^n)
RS	6.62 ± 0.11^b	429.16 ± 11.82^d	0.85 ± 0.02^d	0.01 ± 0.00^a
OFS	6.69 ± 0.09^b	214.27 ± 2.86^a	0.80 ± 0.08^d	0.01 ± 0.01^a
L-RS	6.46 ± 0.07^a	841.53 ± 34.05^f	0.28 ± 0.00^a	2.04 ± 0.27^b
L-OFS	6.49 ± 0.09^a	285.97 ± 2.00^c	0.60 ± 0.07^c	0.09 ± 0.02^a
C-RS	7.23 ± 0.08^c	448.97 ± 11.32^e	0.55 ± 0.09^{bc}	0.10 ± 0.11^a
C-OFS	7.28 ± 0.12^c	231.79 ± 3.87^b	0.47 ± 0.11^b	0.03 ± 0.01^a

Means ± standard deviation in the same column with different letter are significantly different ($P < 0.05$).

lowered to acidic (Kroll, 1984).

3.3.2. Effective particle diameter

The particle diameter is another critical factor in beverages as it may affect the frictional resistance, which influenced the smoothness and terms such as chalky and grainy to describe mouthfeel sensation (Hinds et al., 1997). According to Pathomrungsiyounggul et al. (2013), bovine milk is more favorable in the mouthfeel than other plant-based milk because bovine milk is creamier. Therefore, the particle diameter of soymilk should be controlled to be in the favorable mouthfeel diameter range like bovine milk, which not more than 500 nm (Saeseaw et al., 2005). The particle diameter of soymilk should also be screened to not less than 100 nm because in that range, the mouthfeel is represented as watery (Singer, 1996).

Based on the results presented in Table 3, the grinding method significantly influenced ($P < 0.05$) the particle diameter of soymilk. OFS sample (214.27 nm) showed a smaller particle diameter than RS sample (429.16 nm). This may be attributed to the interaction of soy protein with hydroperoxides as one of the radicals produced by lipid oxidation, resulting in the formation of protein free radicals (Zirlin & Karel, 1969). The occurrence of peroxides on the α-carbon or other carbon of amino acid residue in protein caused an increment in carbonyl content, indicating the changed in amino acid composition thus changing the soy protein structures (Huang et al., 2006). The changed proteins then repack and fold again via non-covalent interactions such as hydrophobic interactions, hydrogen bonds, or electrostatic interactions to form larger aggregates (Huang et al., 2006); as a result, larger particles occurred.

As shown in Table 3, it was found that calcium salts addition significantly increases ($P < 0.05$) the particle diameter of all calcium-enriched soymilk samples. When calcium is added to soymilk, calcium ions neutralize the negatively charged soy protein and leading to aggregation through hydrophobic interactions between neutralized protein molecules (Liu, 1997). Furthermore, the presence of calcium ions caused the combination of protein molecules with oil globule; while carrying round water, the bound globules then aggregate with each other (Ono, 2003) and increase the particle diameter. The use of ca-lactate as a calcium source has a greater impact on particle diameter than ca-carbonate; the same results were also reported by Pathomrungsiyounggul et al. (2010). Lactate-OFS (L-OFS) and lactate-RS (L-RS) have a bigger particle diameter than carbonate-OFS (C-OFS) and carbonate-RS (C-RS). These results are related to the solubility of calcium salts in the liquid medium, where soluble calcium salts show higher effect on the physical stability of calcium-enriched soymilk (Pathomrungsiyounggul et al., 2010). A tremendous increased in particle diameter occurred on L-RS sample (841.53 nm) and far bigger than the diameter ranges for favorable mouthfeel. The emulsion instability of RS was possibly the cause of this significant increase, making it easier for larger oil droplets to bind with soy protein.

3.3.3. Rheological properties

Rheological properties of soymilk and calcium-enriched soymilk were described using a controlled-stress rheometer with viscosity as the main focus of our study. In principle, viscosity is the ratio of shear rates to shear stress. The results of viscosity as a function of shear rate are shown in Fig. 3. It was observed that all samples were shown to have

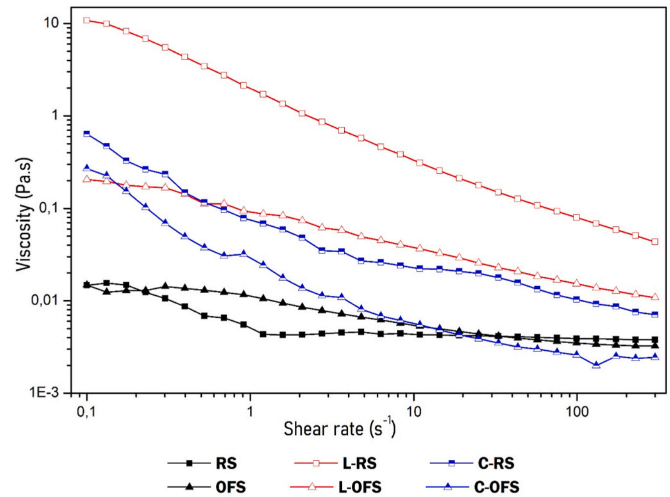

Fig. 3. Changes in rheology properties of soymilk and calcium-enriched soymilk prepared by different grinding methods and calcium salts (RS: regular soymilk, OFS: oxygen-free soymilk, L-RS: lactate-RS, L-OFS: lactate-OFS, C-RS: carbonate-RS, and C-OFS: carbonate-OFS).

shear thinning behaviour, where the viscosity decreased when shear rates increased. Iwuoha and Umunnakwe (1997) reported that the processing method is one factor that could impact the viscosity in soymilk, besides storage temperature, storage time, and total solid. However, this factor did not affect our results because the viscosity of both RS and OFS were not significantly ($P > 0.05$) different. As expected, calcium addition increased the viscosity of all calcium-enriched soymilks due to the interaction of calcium with protein which led to the curd formation (Pathomrungsiyounggul et al., 2007).

Comparing soymilk samples, soymilks enriched with ca-lactate showed a higher increase in viscosity. The same results were also reported by Pathomrungsiyounggul et al. (2010), the addition of ca-lactate at the same concentration with ca-carbonate increased the viscosity of soymilk. This may be attributed to the solubility of calcium salts in the liquid medium. Furthermore, calcium-enriched soymilk prepared from the conventional grinding method showed higher viscosity than others prepared from anaerobic grinding. As the same with particle diameter, the emulsion instability of RS was the cause for this, making it easier for curd formation upon calcium addition.

All the data were fitted to the power-law fluid to understand the rheological behaviour of our samples. According to Table 3, all samples including soymilks and calcium-enriched soymilks showed a flow behaviour index (n values) lower than 1 (0.28–0.85) indicating that they were non-Newtonian fluids (pseudoplastic). A similar observation was also made on the flow consistency index (K values) of all samples, ranging from 0.01 to 2.04. From all of the samples, L-RS showed a significant difference ($P < 0.05$) in n (0.28) and K values (2.04). Forster and Ferrier (1979) explained that the decrease in n value indicates a greater deviation from Newtonian flow behaviour, while the increase in K value indicates a disproportionate increase in viscosity. It is important to control the rheological behaviour of the products in order to produce good quality products.

3.3.4. Microstructures

The microstructures of soymilk and calcium-enriched soymilk prepared by different grinding methods and calcium salts were observed using CLSM. This analysis was performed to identify the distribution of oil and protein droplets, because the behaviour of oil and protein droplets indicates the emulsion stability of soymilk (Durand et al., 2003). Through this analysis, the aggregation that occurred because of calcium addition can also be observed. As shown in Fig. 4, the oil droplet appeared in red while the protein phase appeared in green. The light green hue represented that oil and protein were mixed. It was observed that RS (A) has various oil droplet sizes and is not evenly distributed, while OFS (B) has uniformly distributed oil droplets. These results indicate that soymilk ground with conventional method has unstable emulsion than the anaerobic method. The non-uniform distribution of oil droplets was closely related to the particle diameter (Sivanandan et al., 2010), which was also affected by lipid oxidation during grinding. As reported by Zhou et al. (2019), attractive force such as hydrophobic force (which occurred because of lipid oxidation) promotes the formation of larger aggregates.

The microstructures of calcium-enriched soymilks were also represented in Fig. 4. It was observed that the oil droplets of L-OFS and C-OFS (D, F) were distributed uniformly, even though calcium salts were added. The signs of coagulation were detected in both samples; this was shown by slight aggregation due to the presence of calcium in the samples. Calcium ions in samples can bridge adjacent anionic bodies (two negatively charged particles) so that slight aggregation occurred (Liu et al., 2019). However, coagulation was not practically visible to the naked eye. On the contrary, at L-RS and C-RS (C, E), different results were observed, where protein aggregation and oil droplet coalescence was remarkably shown in the CLSM images, which can be attributed to the emulsion instability of RS. Compared with the C-RS, the matrix structure of the emulsion of L-RS appeared denser, marked with the black background around the matrix; because ca-lactate is more soluble than ca-carbonate, so that ca-protein complex formation was easily occurred and formed a denser structure.

3.4. Calcium bioavailability of calcium-enriched soymilk

In this study, in vitro method was used to estimate the bioavailability of calcium from calcium-enriched soymilk prepared by different grinding methods and calcium salts. Amalraj and Pius (2015) defined calcium bioavailability as the fraction of ingested calcium that can be absorbed and utilized for normal physiological functions or as a reserve. As shown in Fig. 5, the total calcium content of calcium-enriched soymilks was ranging from 1.18 to 1.21 mg/g and had no significant ($P > 0.05$) difference with pasteurized milk (M) as a control sample (1.20 mg/g). After dialysis, the calcium content of C-RS and C-OFS was significantly lower ($P < 0.05$) with 0.28 and 0.27 mg/g, compared to other samples (0.35–0.37 mg/g). Meanwhile, the ca-bioavailability of L-RS and L-OFS (28.99 and 31.21%) have no significant difference ($P > 0.05$) with cow's milk (29.47%) as the control sample and significantly higher ($P < 0.05$) than C-RS (23.73%), and C-OFS (22.35%). The result of ca-bioavailability was in line with the dialyzable calcium because the ca-bioavailability was calculated by dividing the dialyzable calcium with total calcium content in soymilk. The absorption study of calcium-enriched soymilk with cow's milk as control was also studied in Zhao et al. (2005), where calcium-fortified soymilk has equivalent

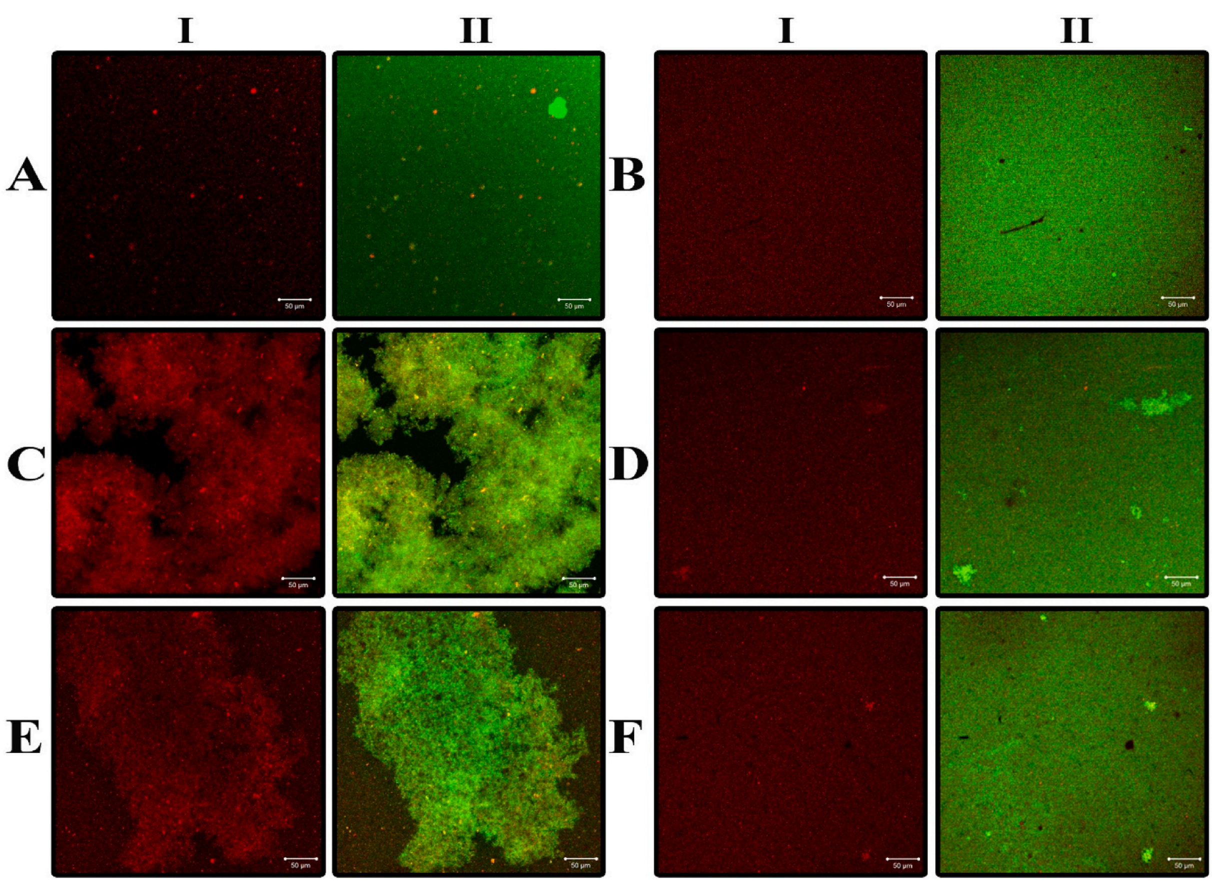

Fig. 4. Microstructures of soymilk and calcium-enriched soymilk prepared by different grinding methods and calcium salts (A: RS, B: OFS, C: L-RS, D: L-OFS, E: C-RS, and F: C-OFS). Oil phase was represented in the red color and protein phase was represented in green color. I showed the oil droplets; II showed the corresponding overlay of oil and protein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

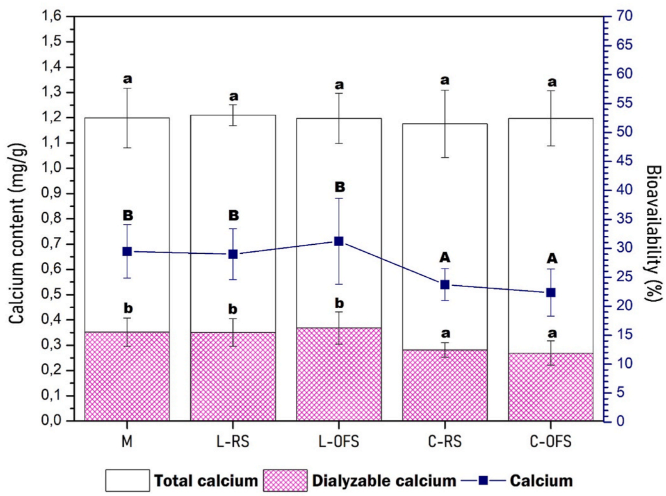

Fig. 5. Total calcium, dialyzable calcium, and calcium bioavailability of milk and calcium-enriched soymilk prepared by different grinding methods and calcium salts (M: cow's milk, L-RS: lactate-RS, L-OFS: lactate-OFS, C-RS: carbonate-RS, and C-OFS: carbonate-OFS).

bioavailability with cow's milk.

This study showed that different grinding methods has no significant effect ($P > 0.05$) on the ca-bioavailability of calcium-enriched soymilk. Simultaneously, different calcium salts have a significant effect ($P < 0.05$) on the ca-bioavailability. From this current investigation, it can be concluded that organic calcium salt such as ca-lactate has higher absorption than inorganic calcium salt such as ca-carbonate. These findings are in accordance with the findings of Goldscher and Edelstein (1996) and Ranjan et al. (2005) for bovine milk and buffalo milk that organic calcium salts are more bioavailable than inorganic salts. Another factor, such as the presence of bile salts during the gastrointestinal process, also influences the availability of calcium (Goss et al., 2007). Expectantly, this study can provide greater insight about calcium bioavailability of calcium-enriched soymilk.

3.5. Sensory evaluation

According to the results presented in Table 4, the highest score for mouthfeel attributes was RS (7.1) and had no significant difference ($P > 0.05$) with other samples except L-RS (4.3). These results were in accordance with the viscosity results, where the addition of ca-lactate to regular soymilk changed the soymilk texture and reduced panellists' preferences. Meanwhile, on the beany flavour attributes, all samples prepared from OFS showed significantly higher ($P < 0.05$) score (6.8–7.1) than other samples prepared from RS (4.8–5.1). These results may be attributed to the reduction of unpleasant beany flavour by avoiding the oxidation of polyunsaturated fatty acid through the anaerobic grinding method. Furthermore, the addition of calcium salts also influenced the panellists' preferences on the bitterness attributes. The addition of ca-carbonate reduced the score to 3.9 and 4.1, which significantly lower ($P < 0.05$) than other samples. The reduction of bitterness score was caused by the undesirable effects of ca-carbonate (soapy and lemony) that may promote astringency and bitter taste in fortified products (Gerstner, 2003; Pathomrungsiyounggul et al., 2013). Meanwhile, the addition of ca-lactate has no significant difference ($P > 0.05$) with unfortified soymilks because ca-lactate has a bland taste (Gerstner, 2003).

Overall, the panellists' acceptability about L-RS showed the lowest score (4.3) and had no significant difference ($P > 0.05$) with C-RS and C-OFS (5.3 and 5.6). A thicker mouthfeel and bitter taste from ca-carbonate caused an unfamiliar sensory sensation in soymilk and decreased the panellist' acceptability. On the other hand, the highest score was found on L-OFS and had no significant difference ($P > 0.05$) with RS and OFS. Based on these results, ca-lactate addition on OFS soymilk produces calcium-enriched soymilk with great acceptability and can improve the quality of soymilk without changing its initial sensory characteristics.

Table 4
Sensory evaluation of soymilk and calcium-enriched soymilk.

	Mouthfeel	Beany flavor	Bitterness	Overall acceptance
RS	7.1 ± 0.99^{b}	4.8 ± 1.55^{a}	6.3 ± 1.83^{b}	6.6 ± 1.35^{bc}
OFS	6.9 ± 1.45^{b}	7.1 ± 1.29^{b}	5.8 ± 1.03^{ab}	6.7 ± 0.48^{bc}
L-RS	4.3 ± 1.25^{a}	5.0 ± 2.00^{a}	5.8 ± 2.90^{ab}	4.3 ± 1.42^{a}
L-OFS	6.5 ± 1.27^{b}	6.9 ± 0.99^{b}	6.3 ± 1.77^{b}	7.0 ± 1.76^{c}
C-RS	6.1 ± 1.45^{b}	5.1 ± 1.60^{a}	4.1 ± 1.91^{a}	5.3 ± 2.11^{ab}
C-OFS	6.1 ± 1.73^{b}	6.8 ± 1.32^{b}	3.9 ± 2.13^{a}	5.6 ± 1.51^{abc}

Means ± standard deviation in the same column with different letter are significantly different ($P < 0.05$).

4. Conclusion

In summary, different grinding methods and calcium salts addition influenced soymilk properties and improved the quality. The use of the anaerobic grinding method decreased the oxidative degradation of both lipid and protein in soymilk by limiting oxygen as the reactant for the enzymatic reaction catalysed by LOX. As the result of anaerobic grinding method, OFS has lower beany flavour than RS, making it possible to produce soymilk with lower off-flavour and increase its sensory acceptance. Simultaneously, the addition of ca-lactate reduced soymilk pH, while ca-carbonate increased the pH. The addition of calcium salts also created protein aggregation and coalescence of oil droplets when CLSM visualized the microstructure of all samples. The emulsion instability of RS sample was also shown through the non-uniform of oil droplets on CLSM visualisation. This emulsion instability was the cause for the increase in particle diameter and viscosity of soymilk when calcium salts were added. The solubility of ca-lactate and ca-carbonate was also influenced soymilk stability. It was observed that ca-lactate has a higher dissolution rate than ca-carbonate, either in water or soymilk medium. Based on the results, it is recommended to use ca-lactate as a calcium source in soymilk because it has equivalent calcium bioavailability with cow's milk. Combining the anaerobic grinding method with ca-lactate addition may be an effective way to produce soymilk with good sensory acceptability, good stability, and high calcium bioavailability. It is expected that this study can provide fundamental information to the production of good quality soymilk.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT author contribution statement

Victor Christian Kaharso: Conceptualization, Formal Analysis, Investigation, Writing – Original Draft.

Bertrand Muhoza: Writing – Review & Editing, Data Curation.

Xiangzhen Kong: Writing – Review & Editing, Methodology.

Yufei Hua: Conceptualization, Supervision, Funding Acquisition.

Caimeng Zhang: Project Administration, Resources.

Data availability statement

All data generated or analysed during this study are included in this manuscript.

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