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The Quality and Flavor Changes of Different Soymilk and Milk Mixtures Fermented Products during Storage

Lina Zong [1], Maolin Lu [2], Wenqiong Wang [2], Yunchao Wa [2], Hengxian Qu [2], Dawei Chen [2], Yang Liu [2], Yi Qian [2], Qingyang Ji [2] and Ruixia Gu [2,*]

[1] College of Food Science and Engineering, Yangzhou University, Yangzhou 225000, China
[2] Jiangsu Key Laboratory of Dairy Biotechnology and Safety Control, Yangzhou University, Yangzhou 225000, China
* Correspondence: guruixia1963@163.com

Abstract: This study explored the effects of two mixed fermentation methods: one was fermenting a soymilk and milk mixture by a lactic acid bacteria fermenting agent at 0.1 g/kg and 42 °C until the acidity was 70 °T, which was set as the MFSM method, and the other was fermenting milk alone by lactic acid bacteria at 42 °C for 12 h, placing it in a 4 °C refrigerator after acidification for 24 h and then mixing it with soymilk at a 1.5:1 ratio and storing the mixture at 4 °C, which was set as the SMFSM method. The quality and flavor of the soymilk and milk mixture products were investigated on the 0th, 15th and 30th days during storage. The changes in acidity, pH, number of viable bacteria, viscosity, water-holding capacity, texture, rheological properties, sensory quality and volatile flavors were determined. The results showed that compared with the fermented soymilk and milk mixtures under the MFSM method, the samples of fermented soymilk and milk mixtures under the SMFSM method showed a significant slowdown of acidification during storage, so that the sensory quality of the products was almost unaffected by acidity on the 30th day of storage. Furthermore, the number of viable bacteria was greater than 7 log cfu/mL. The water holding capacity did not change significantly until the 30th day. There was also no whey precipitation, indicating good stability. The samples in SMFSM mode had higher aromatic contents and beans during storage than the fermented soymilk and milk mixtures in MFSM mode. The rich variety of volatile flavors and the presence of acetoin, 2-heptanone, and (E,E)-3,5-octadien-2-one throughout the storage period allowed the samples to maintain a good sensory flavor during storage.

Keywords: mixed fermentation methods; fermented soymilk and milk mixtures; storage period; flavor; sensory quality

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1. Introduction

Soymilk is an aqueous extract of soybeans and is a good source of high-quality protein and many micronutrients, such as isoflavones, saponins, oligosaccharides, dietary fiber and phytoinositol [1,2]. Moreover, it is low in fat and an economical source of protein, which has an important place in both Eastern and Western diets [3,4]. The fermentation of soymilk by lactic acid bacteria improves its physicochemical and organoleptic properties as well as its nutritional value. However, there are some problems in using lactic acid bacteria to ferment soymilk, such as a low acid production rate, slow growth of lactic acid bacteria and odor production. In the face of these problems, some researchers have provided solutions. Wang et al. [4] used *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* to inoculate soymilk in a 1:1:1 ratio. The pH was reduced to below 4.3 after 8 h of incubation at 37 °C and 43 °C, and the content of esters and ketones in fermented soymilk increased, resulting in more aroma components in fermented soymilk. A strain of *Lactobacillus harbinensis M1* was screened by Yinzheng et al. [5], which led to a high yield of 2,3-butanedione and acetoin and improved the organoleptic quality of fermented soymilk. However, further studies on the quality and flavor improvement of fermented soymilk are still needed. Mixed fermentation of soy and cow's milk is a good option. Both soy and cow's milk are accepted by a wide range of consumers, and mixed fermentation provides several advantages, such as improving the flavor of fermented soymilk while reducing the relatively high cost of cow's milk, and in terms of nutrition, they are highly compatible. Mitsuru Yoshida et al. [6] found that the samples fermented with traditional lactic acid bacteria starter (*Lactobacillus deuterium* subsp. *bulgaricum* and *Streptococcus thermophilus*) and *Bifidobacterium* in the mixture of soymilk and milk have better taste than those fermented with soymilk alone, mainly due to reducing the production of hexanal and valeraldehyde which cause undesirable flavors.

The texture and organoleptic quality of fermented dairy products are decisive factors in the consumer's choice during the purchase process [7,8]; therefore, many researchers have provided different solutions to improve the texture and organoleptic quality of fermented soymilk. At present, most studies have mainly investigated the effects of different microbial combinations; for example, cofermentation using *Lactobacillus plantarum* and *Bifidobacterium bifidum* with traditional fermenters can improve the cohesiveness of fermented milk [9]. In terms of the mixing ratios of soymilk and milk, the texture was more popular in the sample of 75% milk mixed with 25% soymilk [10,11], and the addition of prebiotics affected the texture and flavor of fermented soymilk [12,13]. Some studies have shown that the texture and sensory quality of fermented soymilk can be improved by different mixed fermentation methods. Different fermentation mixes may affect the physicochemical, textural, sensory and flavor substances of fermented soymilk, and these food quality attributes play a critical role in the overall acceptability and stability of the product during storage [14].

Most studies have examined the effect of mixed fermentation of soymilk on the texture and flavor of fermented soymilk. Therefore, the main objective of this study was to compare the effects of two mixed fermentation methods on fermented soymilk and milk mixtures. In this study, the ratio of milk and soymilk mixture was 1.5:1. One method was to mix milk and soymilk in a ratio of 1.5:1, inoculated with a lactobacillus starter, incubated at 42 °C until 70 °T, and then placed in a 4 °C refrigerator for storage. The other method was to ferment milk alone first, and after the fermentation of milk to 110 °T, it was acidified for 24 h, mixed with soymilk at a ratio of 1.5:1, stirred well and then refrigerated at 4 °C. The changes in acidity, pH, viable bacteria, viscosity, water-holding capacity, texture, rheology, volatile flavor substances and sensory scores of the samples prepared by the two methods were compared on the 0th, 15th and 30th days.

2. Materials and Methods

2.1. Materials

Soybeans were purchased from Tmall supermarkets (Yangzhou, Jiangsu, China). M17 and MRS media were obtained from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co. (Qingdao, Shandong, China). Lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were obtained from Jiangsu Key Laboratory of Dairy Biotechnology and Safety Control (Yangzhou, Jiangsu, China).

2.2. Methods

2.2.1. Preparation of Soymilk and Milk

The soybeans were soaked in 0.3% $NaHCO_3$ aqueous solution for 16 h after washing, and boiled at 100 °C for 8 min after cleaning. Then, the soymilk was ground with distilled water at 90 °C (the ratio of material to liquid was 1:8). After high-pressure homogenization (20 MPa), the sample was heat treated at 95–100 °C for 10 min and cooled.

Milk was prepared by first adding 8% sucrose to 11.5% whole milk. Then, the mixture was evenly mixed, homogenized at 20 MPa, heat treated at 95 °C for 10 min, cooled to room temperature and set aside.

2.2.2. Preparation of Fermented Soymilk and Milk Mixtures

Soymilk and milk were mixed at a ratio of 1.5:1 and stirred evenly, heat treated at 95 °C for 10 min, cooled to 42 °C, inoculated with a lactic acid bacteria fermenting agent at 0.1 g/kg, and fermented at 42 °C until the acidity was 70 °T (abbreviated as MFSM). Milk alone was fermented for 12 h in a 42 °C incubator, the acidity of milk was 100 °T, removed and placed in a 4 °C refrigerator after acidification for 24 h, the acidity is 110 °T, and mixed with soymilk at a 1.5:1 ratio; then, the mixture was stirred well, the acidity of the sample was 70 °T and placed at 4 °C (abbreviated as SMFSM).

2.2.3. Physicochemical Analysis

The acidity was determined according to the determination method of acidity in GB5009.239-2016 and the phenolphthalein indicator method. The pH was measured with a precision pH meter (pH S-3E precision pH meter, Leici Instrument Factory, Shanghai, China). Viscosity measurements of fermented soymilk and milk mixtures were taken at 25 °C and 30 rpm (spindle number 4) with a viscometer (RVDV-II+). Each measurement was taken three times. The determination of the water-holding capacity of fermented soymilk and milk mixtures was conducted as follows: A lot of 10 g of fermented soymilk and milk mixtures sample was packed in a centrifuge tube and centrifuged (Legend mach1.6R, Thermo Fisher Technology Co., Ltd., New York, NY, USA) at 4000× *g* for 10 min at 4 °C to calculate its water-holding capacity.

2.2.4. Microbiological Analysis

The number of microorganisms in the samples was determined by the plate counting method. Among them, *Streptococcus thermophilus* was cultured in the M17 medium and *Lactobacillus bulgaricus* was cultured in the MRS medium. The number of viable bacteria was determined according to GB4789.2-2016.

2.2.5. Volatile Aromatic Compound Analyses

Volatile aromatic compounds were determined on the 0th, 15th and 30th days of storage. An aliquot of 5.00 g of fermented sample was added to a headspace bottle. The test conditions referred to Ref. [15] with slight modifications.

GC: An HP-5 capillary column (30 mm × 0.25 mm, 0.25 µm) was used. Adopting the programmed heating method, the initial temperature was 35 °C for 5 min, increased to 140 °C at a rate of 5 °C/min for 2 min, increased to 250 °C at a rate of 10 °C/min, and held for 3 min. The temperature of the vaporization chamber was 250 °C. The carrier gas was He, and the flow rate was 1.0 mL/min. No split injection was performed.

MS: An electron ionization source was used. The electron energy was 70 EV. The ion source temperature was 230 °C, and the mass scanning range was *m/z* 35~500. The emission current was 100 µA.

SPME: The aging temperature of the extraction head was 250 °C, the aging time was 20 min, the equilibrium temperature was 50 °C, the equilibrium time was 45 min, and the desorption was 3 min at 250 °C.

2.2.6. Sensory Analysis

The sensory evaluation team was composed of 10 graduate students from the dairy laboratory. All the graduate students on the team received sensory training on fermented milk and fermented soymilk. The following odor characteristics were evaluated: sour, fatty, aromatic, beany, woody and grassy flavors. The intensity of each attribute was recorded on a seven-point scale from 1 (low intensity) to 7 (high intensity) [16]. The fermented soymilk and milk mixtures were scored on the 0th, 15th and 30th days of storage.

2.2.7. Textural Properties

The texture of the fermented soymilk and milk mixtures was determined using a texture analyzer (TMS-Pro, TMS Corp., Henderson, NV, USA). Referring to the method developed by Cao et al. [17], the A/BE probe was installed, the prepressure speed was set at 1.0 mm/s, the post pressure speed was set at 1.0 mm/s, the starting pressure was 0.1 N, the height was 70 mm and the type variable was 50%. A glass bottle containing 100 mL of the sample was placed on the shelf, and its hardness, adhesiveness, cohesiveness, stickiness and chewiness were measured.

2.2.8. Rheological Properties

Frequency scanning of fermented soymilk and milk mixtures was performed using a rotational rheometer (Malvern Kinexus Pro, Malvern Instruments Ltd., Worcestershire, UK). Two milliliters of the sample was placed on the operating table, and a frequency sweep was performed at 0.5% strain in the linear viscoelastic region (frequency: 0.1–10 Hz) [18]. The energy storage modulus (G') and loss modulus (G'') of the samples during storage were determined.

2.3. Statistical Analyses

Microsoft Excel 2021, Statistics 20 and Origin Pro2021 were used for statistical analysis. The test results are expressed as the mean ± standard deviation. When $p < 0.05$, the test results are significant.

3. Results and Discussion

3.1. Acidity and pH of Fermented Soymilk during Storage

In fermented dairy products, post acidification will lead to serious product acidity and whey release, which has a great impact on the quality of fermented dairy products [19]. The changes in acidity and pH of fermented soymilk and milk mixtures prepared by the two mixed fermentation methods during storage are shown in Figure 1. The samples under both methods were stored at 70 ± 2 °T at the end of the fermentation, and then stored in a refrigerator at 4 °C. The acidity and pH were measured on the 0th, 15th and 30th days. The acidity of the samples is shown in Figure 1a. The acidity of both samples showed an increasing trend from 0 to 30 days, and the acidity of the samples under the MFSM method was significantly higher than that of the samples under the SMFSM method. The post acidification capacity of the samples under the MFSM method was greater. This was probably related to the fact that the carbohydrates and nutrients remaining in the mixed matrix prompted the lactic acid bacteria to continue fermentation and acid production [20], which enabled a large increase in MFSM sample acidity. The SMFSM samples have less change in acidity during 0–30 days. This is because the acidity of using lactic acid bacteria to ferment milk needs to reach 110 °T to meet the acidity of mixed fermented soymilk and milk mixtures at 70 °T. Due to the large consumption of sugars in fermented milk and the extremely low pH, the microorganism growth rate decreases. Therefore, the cell-growth rate is still slow after the fermented milk is mixed with soymilk. Therefore, the acidification degree of SMFSM samples after the storage is weak [21]. The pH changes of the samples during storage are shown in Figure 1b. The pH of the samples under both mixed fermentation methods showed a decreasing trend, contrary to the trend of acidity. The pH decreased from 4.38 to 4.01 for the MFSM samples and from 4.46 to 4.21 for the SMFSM samples. During storage, lactose is dissociated into lactic acid, which helps to decrease the pH. In addition, the buffering capacity of casein determines the pH fluctuation during storage [22]. Therefore, the samples of the two mixed fermentation methods have different degrees of pH reduction. Throughout the storage period, the pH of the MFSM samples was always lower than that of the SMFSM samples.

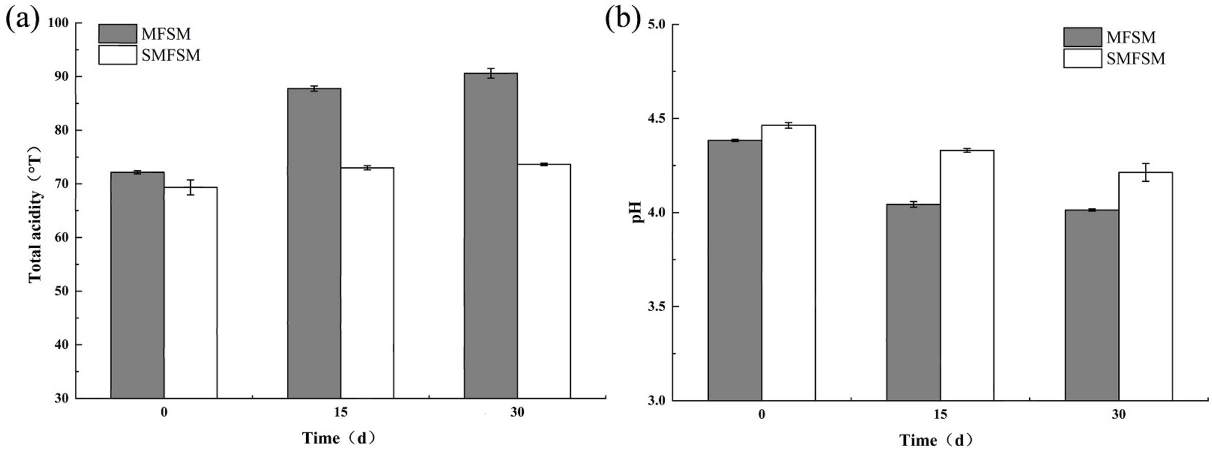

Figure 1. Effects of two mixed fermentation methods on the acidity and pH of samples during storage: (**a**) the change in acidity during storage and (**b**) the change in pH during storage.

3.2. Microbiology of Fermented Soymilk during Storage

The live bacteria count of fermented foods is very important because it is beneficial to reach a certain number of live bacteria [7]. However, the probiotic activity in fermented milk is affected by many factors, such as the pH and lactic acid and acetic acid concentrations [23]. The viable bacteria count of the fermented soymilk and milk mixtures prepared by both blends was maintained at 10^7 cfu/mL during refrigeration, as shown in Figure 2. Both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* of the MFSM samples showed an increasing trend followed by a decreasing trend during storage. On the 15th day, the highest number of viable bacteria was observed. A trend of increasing and then decreasing *Streptococcus thermophilus* in the SMFSM samples, similar to the research results of MANORAMA K. *Streptococcus thermophilus* has the ability to produce exopolysaccharides and flavor compounds [2]. while *Lactobacillus delbrueckii* subsp. *bulgaricus* showed a decreasing trend throughout the storage period. Lactic acid bacteria starter cultures are diverse, and there are great differences in acid production and extracellular polysaccharide production. During storage at 4 °C, *Lactobacillus delbrueckii* subsp. *bulgaricus* has a high-protein hydrolysis and metabolism ability, and with the production of lactic acid, it has a great impact on the acidification of fermented soymilk [24]. These results demonstrate that different mixed fermentation methods affect the growth of lactic acid bacteria during storage.

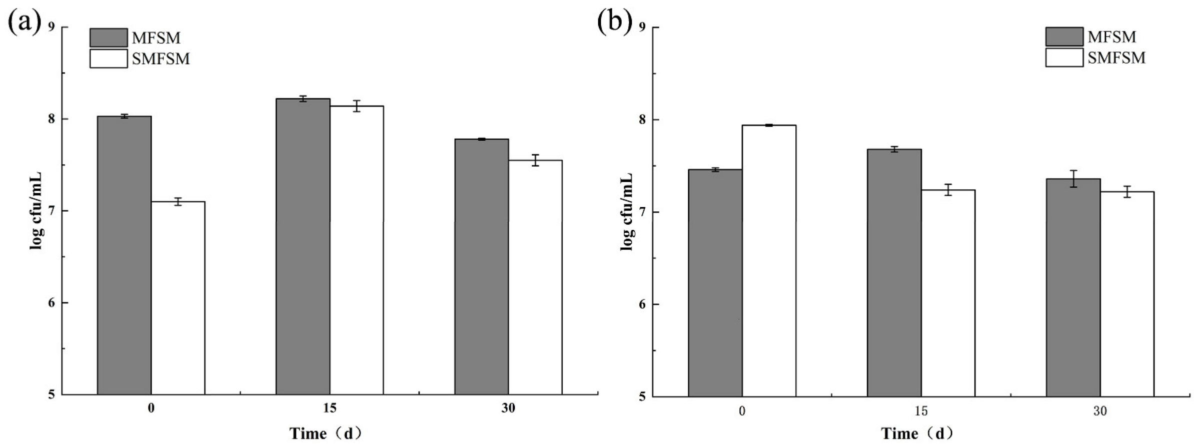

Figure 2. Effect of two mixed fermentation methods on the number of viable bacteria during storage: (**a**) the change in *Streptococcus thermophilus* during storage and (**b**) the change in *Lactobacillus delbrueckii* subsp. *bulgaricus* during storage.

3.3. Viscosity and Water-Holding Capacity of Fermented Soymilk during Storage

As shown in Figure 3a, the viscosity of fermented soymilk and milk mixtures obtained using the two mixed fermentation methods showed a downward trend during the whole storage period, which may be related to the high activity of microorganisms. The degradation of peptides by *Lactobacillus delbrueckii* subsp. *bulgaricus* promotes the growth of Streptococcus thermophilic, produces more lactic acid, and affects the interaction of protein networks, thus affecting the viscosity of products [25,26]. Figure 3b shows the change in the water-holding capacity of fermented soymilk and milk mixtures with two mixed fermentation methods during storage. The change in the water-holding capacity of MFSM samples is consistent with the changing trend of their viscosity, while the water-holding capacity of SMFSM samples increased on the 15th day, possibly due to the increased cross-linking of curd [27]. It may also be that EPS is produced, which enhances the stability of the protein gel network [28]. The viscosity and water-holding capacity of the MFSM sample are significantly higher than those of the SMFSM sample ($p < 0.05$) because the casein of the MFSM sample and the white soymilk aggregate at the same time, which will be more evenly dispersed in the whole protein network [29]. For the SMFSM sample, casein aggregates first, and then soymilk chelates into the already-formed casein network micelle. Therefore, the gel network obtained may not be uniform, so the MFSM sample has a high viscosity and water-holding capacity [29].

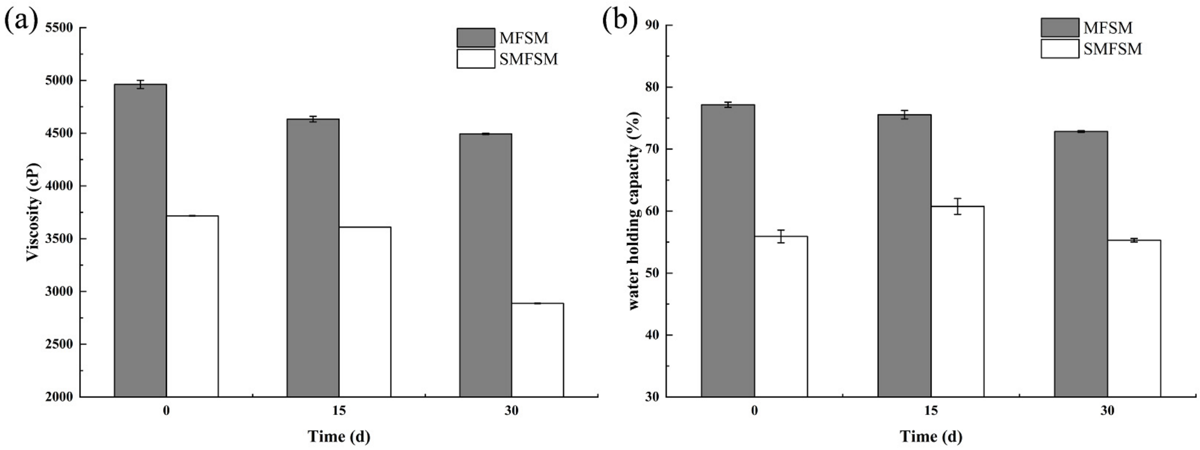

Figure 3. Effect of two mixed fermentation methods on the viscosity and water-holding capacity of fermented soymilk and milk mixtures during storage: (**a**) the change in viscosity during storage and (**b**) shows the change in water-holding capacity during storage.

3.4. Changes of Volatile Components during Storage

The volatile components of fermented soymilk and milk mixtures obtained using the two mixed fermentation methods during storage are shown in Table 1, and a total of 55 major volatile flavor substances were detected. These compounds included 10 ketones, nine acids, eight aldehydes, nine alcohols, six esters and 13 other compounds. The MFSM samples contained 23 volatile compounds on the 0th day, 26 volatile compounds on the 15th day and 16 volatile compounds on the 30th day. The SFMSM samples contained 25 volatile compounds on the 0th day, 22 volatile compounds on the 15th day and 1 volatile compounds on the 30th day. It can be seen from the results that different mixed fermentation methods have a great influence on both the types and contents of volatile substances during the storage of fermented soymilk and milk mixtures.

Table 1. Effect of different mixed fermentation methods on the volatile flavors of fermented soymilk during storage.

	Time	English Name	Molecular Formula	MFSM			SMFSM		
				M_0	M_{15}	M_{30}	S_0	S_{15}	S_{30}
1	2.47	2,3-Butanedione	$C_4H_6O_2$	11.75	1.28	-	-	-	-
2	2.50	Deoxyspergualin	$C_{17}H_{37}N_7O_3$	-	-	1.21	-	-	-
3	2.55	Cystine	$C_6H_{12}N_2O_4S_2$	-	-	-	1.5	-	-
4	3.69	2-Pentanone	$C_5H_{10}O$	-	-	-	1.05	-	-
5	3.89	Acetaldehyde	C_2H_4O	-	-	-	-	-	0.68
6	4.19	Acetic acid	$C_2H_4O_2$	-	-	-	2.83	0.75	1.41
7	4.22	2,3-Pentanedione	$C_5H_8O_2$	2.22	0.98	1.16	-	-	-
8	4.43	Acetoin	$C_4H_8O_2$	11.81	4.21	5.4	7.12	3.28	0.53
9	6.62	1-Pentanol	$C_5H_{12}O$	1.57	1.14	1.25	2.96	0.16	0.74
10	7.06	9-Octadecen-12-ynoic acid, methyl ester	$C_{19}H_{32}O_2$	0.24	-	-	-	-	-
11	7.71	2-methyl-3-Pentanol	$C_6H_{14}O$	4.79	-	-	-	-	-
12	7.76	(R)-2-Methyl-2,4-pentanediol	$C_6H_{14}O_2$	-	2.73	4.49	-	-	-
13	8.19	2-Hydroxy-3-pentanone	$C_5H_{10}O_2$	-	0.93	-	-	-	-
14	9.05	1,2-bis[(4-amino-3-furazanyl)oxy]- Ethane	$C_6H_8N_6O_4$	-	-	-	2.05	-	-
15	10.67	1-Hexanol	$C_6H_{14}O$	8.11	5.81	4.76	-	2.38	3.57
16	11.26	2-Heptanone	$C_7H_{14}O$	7.77	2.78	-	16.35	4.1	0.87
17	13.87	(Z)-2-Heptenal	$C_7H_{12}O$	-	0.45	-	0.72	0.39	-
18	14.03	Benzaldehyde	C_7H_6O	-	1.18	3.68	-	0.62	-
19	14.46	1-Heptanol	$C_7H_{16}O$	1.25	-	-	1.26	0.48	-
20	14.55	Formic acid, heptyl ester	$C_8H_{16}O_2$	-	0.48	-	-	-	-
21	15.00	2-pentyl-Furan	$C_9H_{14}O$	1.01	-	-	-	0.21	-
22	15.53	Hexanoic acid	$C_6H_{12}O_2$	1.74	1.61	-	3.83	1	-
23	15.84	(E,E)-2,4-Heptadienal	$C_7H_{10}O$	-	-	-	1.19	-	-
24	16.72	cis-11-Eicosenoic acid	$C_{20}H_{38}O_2$	-	-	-	0.37	-	-
25	17.21	3-ethyl-5-(2-ethylbutyl)- Octadecane	$C_{26}H_{54}$	0.24	-	-	-	-	-
26	17.24	2,6,10-trimethyl-Tetradecane	$C_{17}H_{36}$	-	0.19	-	-	-	-
27	17.42	(E)-2-Octenal	$C_8H_{14}O$	-	0.29	-	0.47	0.24	-
28	17.79	(Z)-3-Nonen-2-ol	$C_9H_{18}O$	-	-	-	0.89	-	-
29	17.87	(Z)-2-Octen-1-ol	$C_8H_{16}O$	-	0.19	-	-	0.12	-
30	17.91	1-Octanol	$C_8H_{18}O$	1.34	0.65	-	1.12	0.36	-
31	18.45	2-Nonanone	$C_9H_{18}O$	4.40	1.53	1.74	7.38	2.78	1.36
32	18.58	(E,E)-3,5-Octadien-2-one	$C_8H_{12}O$	1.16	0.39	0.56	1.86	0.24	0.34
33	18.64	Isopentyl 3-hydroxy-2-methylenebutanoate	$C_{10}H_{18}O_3$	-	0.25	-	-	-	-
34	18.72	3-ethyl-5-(2-ethylbutyl)- Octadecane	$C_{26}H_{54}$	0.17	0.13	-	-	0.19	-
35	18.73	6-methyl-Octadecane	$C_{19}H_{40}$	-	0.29	-	0.7	-	-
36	20.61	2,6,10-trimethyl- Dodecane	$C_{15}H_{32}$	0.62	0.21	-	1.32	0.16	-
37	21.08	1-Nonanol	$C_9H_{20}O$	0.74	0.43	-	1.03	-	-
38	21.45	Octanoic acid	$C_8H_{16}O_2$	1.02	-	-	-	-	0.5
39	21.84	Dodecane	$C_{12}H_{26}$	0.40	-	-	0.55	-	-
40	22.06	Decanal	$C_{10}H_{20}O$	0.38	0.16	-	0.68	-	-
41	22.41	(2-phenyl-1,3-dioxolan-4-yl) methyl ester, cis-9-Octadecenoic acid	$C_{28}H_{44}O_4$	-	-	1.78	-	-	-
42	22.51	Ethyl maltol	$C_7H_8O_3$	-	-	4.8	-	-	-
43	22.61	3-ethyl-5-(2-ethylbutyl)-Octadecane	$C_{26}H_{54}$	-	-	0.65	0.31	-	-
44	22.84	3-(octadecyloxy) propyl ester Oleic acid	$C_{39}H_{76}O_3$	-	-	0.3	-	-	-
45	23.20	2,4-dimethyl-Benzaldehyde	$C_9H_{10}O$	-	-	1.72	-	-	1.39
46	24.20	Nonanoic acid	$C_9H_{18}O_2$	-	-	-	0.4	0.3	-
47	24.58	2-Undecanone	$C_{11}H_{22}O$	-	-	0.58	-	-	-
48	25.32	(E,E)-2,4-Decadienal	$C_{10}H_{16}O$	0.39	0.56	-	-	0.98	-
49	27.63	3-ethyl-5-(2-ethylbutyl)- Octadecane	$C_{26}H_{54}$	0.24	-	-	-	0.12	-
50	30.28	Dodecalactone	$C_{12}H_{22}O_2$	0.24	-	-	-	-	-
51	30.34	2-Tridecanone	$C_{13}H_{26}O$	-	0.22	-	-	-	-

Table 1. *Cont.*

	Time	English Name	Molecular Formula	MFSM			SMFSM		
				M_0	M_{15}	M_{30}	S_0	S_{15}	S_{30}
52	30.54	Butylated Hydroxytoluene	$C_{15}H_{24}O$	-	-	8.43	-	-	6.83
53	33.94	3-ethyl-5-(2-ethylbutyl)-Octadecane	$C_{26}H_{54}$	-	-	-	-	0.17	-
54	35.58	Isopropyl myristate	$C_{17}H_{34}O_2$	-	-	-	-	0.23	-
55	36.04	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	-	-	-	1.49	-	-

M is the abbreviation for MFSM, S is the abbreviation for SMFSM and the corner notation indicates the storage time. The content of volatile flavor substances is expressed by the concentration (μg/L).

Ketones are important flavor substances in fermented milk, and acetoin, 2-nonanone and (E,E)-3,5-octadien-2-one were detected in both MFSM and SMFSM samples during storage, and the content of flavor substances which were lower on the 15th and 30th days than on the 0th day. The decrease in acetoin results in a consequent decrease in the creamy aroma of fermented soymilk and milk mixtures. 2,3-Butanedione was detected in the MFSM samples at 11.75 μg/L on the 0th day, which decreased to 1.28 μg/L on the 15th day and was not detected on the 30th day. This may be due to the conversion of 2,3-butanedione to small amounts of acetoin and other flavor compounds. 2,3-Pentanedione was also detected in the MFSM sample, while it was not detected in the SMFSM sample, which could be due to the difference caused by the different mixing fermentation methods. A significant amount of 2-heptanone was also detected in the MFSM and SMFSM samples. 2-Heptanone is the main source of cheese aroma, which imparts a mellow flavor to fermented soymilk, making it preferable. The 2-heptanone content decreased with time. However, the 2-heptanone content of SMFSM samples was always higher than that of MFSM samples under the same storage time, and the presence of 2-heptanone improved the aroma of SMFSM samples. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* make a great contribution to the volatile flavor of fermented milk. Lactobacillus can produce pyruvic acid by metabolizing lactose, and pyruvic acid can produce ketones [30]. SMFSM samples are prepared by lactic acid bacteria alone fermenting milk, acidified and then mixed with soymilk. Lactobacillus can metabolize more lactose when fermenting in a separate milk system, so more ketones may be produced. There are many ketones in the sample after being mixed with soymilk, but no literature shows which ketone is specific. Therefore, it is speculated that 2-heptanone may be one of them.

Hexanoic acid was detected in the MFSM and SMFSM samples on the 0th and 15th days and disappeared from both mixed fermentation methods samples on the 30th day, probably due to its binding to esters by interaction with alcoholic compounds [16,31]. Acetic acid was detected in the SMFSM samples at 2.83 μg/L, 0.75 μg/L and 1.41 μg/L at the 0th, 15th and 30th days, respectively. The acetic acid content is more appropriate in the range of 0.5–18.8 μg/L, which gives a pleasant tart flavor to fermented soymilk and milk mixtures. Small amounts of other acids, such as octanoic and nonanoic acids, were also detected in the samples from both mixed fermentation methods.

Aldehydes contribute more value to the flavor of fermented soymilk [15]. The MFSM and SMFSM samples showed no significant pattern of changes in the detected aldehydes during the storage period. The presence of only a small amount of (E,E)-2,4-decadal gives a large off-flavor to the sample due to its extremely low threshold. For the MFSM samples, 0.39 μg/L and 0.56 μg/L (E,E)-2,4-decadal were detected on the 0th day and 15th day, respectively. For the SMFSM sample, 0.98 μg/L was detected on the 15th day, and its presence reduced the aromatic taste of fermented soymilk and milk mixtures. The presence of (E)-2-octenal gives a pleasant aromatic flavor to fermented soymilk and milk mixtures.

The alcohols detected in the fermented soymilk and milk mixtures from both mixed fermentation methods during storage were 1-pentanol, 2-methyl-3-pentanol, (R)-2-methyl-2,4-pentanediol, 1-hexanol, 1-heptanol, (Z)-3-nonen-2-ol, (Z)-2-octen-1-ol, 1-octanol and 1-nonanol. Alcohols generally have higher thresholds and less influence on the flavor of fermented soymilk and milk mixtures. During storage, higher levels of 1-hexanol were detected in the MFSM samples and its presence imparted a fatty taste to fermented soymilk and milk mixtures, while it was not detected on the 0th day for the SMFSM samples. However, 1-hexanol was detected during subsequent storage, but its levels were all lower than those of MFSM samples.

Ester compounds were detected in smaller amounts and did not vary regularly. Among the other compounds, alkanes enriched the aromatic flavor of fermented soymilk and milk mixtures, while the presence of 2-pentylfuran gave an undesirable flavor to fermented soymilk and milk mixtures; therefore, the sensory scores of MFSM samples were lower than those of SMFSM samples on the 0th day.

3.5. Principal Component Analysis, Cluster Analysis and Sensory Analysis

Principal component analysis explains the internal relationship between multiple variables by a few principal components to provide a more objective response to the type and content of substances in the sample. The data of volatile flavor substances in fermented soymilk and milk mixtures were processed by principal component analysis, which is a feasible and scientific method. During storage, a higher correlation was indicated by the closer sample points of the fermented soymilk and milk mixtures from the two mixed fermentation methods with the different types of flavor substances in the load diagram. The principal component analysis of the volatile species of the fermented soymilk and milk mixtures from different mixed fermentation methods during storage is shown in Figure 4a. S_0 was highly correlated with ketones, acids and esters, M_0 and M_{15} were highly correlated with alcohols, and M_{30} was highly correlated with aldehydes and other compounds, while S_{30} and S_{15} did not show a correlation with the compounds. The principal component analysis of the main volatiles of the fermented soymilk and milk mixtures from different mixed fermentation methods during storage is shown in Figure 4b. Compounds that are highly correlated with M_0 include acetoin, 2,3-butanedione, 2-methy-3-pentanol, 1-heptanol, 1-octanol, 1-nonanol and decanal. Acetoin and butanedione provide powerful creamy and milky flavors to the products. However, excessive amounts of 2,3-butanedione can unbalance the aroma of fermented soymilk [4]. Compounds highly correlated with S_0 include 1-pentanol, 2-pentanone, 2-heptanone, hexanoic acid, (E)-2-octenal, 2-nonanone, (E,E)-3,5-octadien-2-one and nonanoic acid. Compounds highly related to M_{15}, M_{30}, S_{15} and S_{30} are benzaldehyde and 2-undecanone. Benzaldehyde produces a bitter almond taste and brings out the undesirable flavor of fermented soymilk and milk mixtures, thus reducing its sensory score.

The clustering analysis of the main volatile flavor substances of the fermented soymilk and milk mixtures of the two mixed fermentation methods during storage is shown in Figure 4e,f, where the fermented soymilk and milk mixtures of the two mixed fermentation methods had more similar substances clustered into one branch on the 0th and 15th days. On the 30th day, the samples of both mixed fermentation methods showed a decrease in species, while the content showed different degrees of variation. Higher levels of 1-hexanol, 2-nonanone and 1-pentanol were detected in the samples from both mixed fermentation methods. These three substances can bring undesirable flavors such as grassiness and fatty taste, to fermented soymilk and milk mixtures. Throughout the storage period, the MFSM sample showed a decreasing trend in 1-hexanol content, therefore resulting in a grassy flavor, while the SMFSM sample showed an increasing trend in n-hexanol content, but the content was still significantly lower than that of the MFSM sample; therefore, fewer bad flavors were produced. However, benzaldehyde was detected in the MFSM sample and exacerbated the undesirable flavor of fermented These three substances can bring undesirable flavors such as grassiness and fatty taste, to fermented soymilk and milk mixtures, while 2-heptanone was detected in the SMFSM sample to impart a creamy aroma and enhance the aromatic flavor of fermented soymilk and milk mixtures. In summary, the aroma and bean flavor of fermented soymilk and milk mixtures from both mixed fermentation methods decreased with longer storage time, but the SMFSM samples had better flavor.

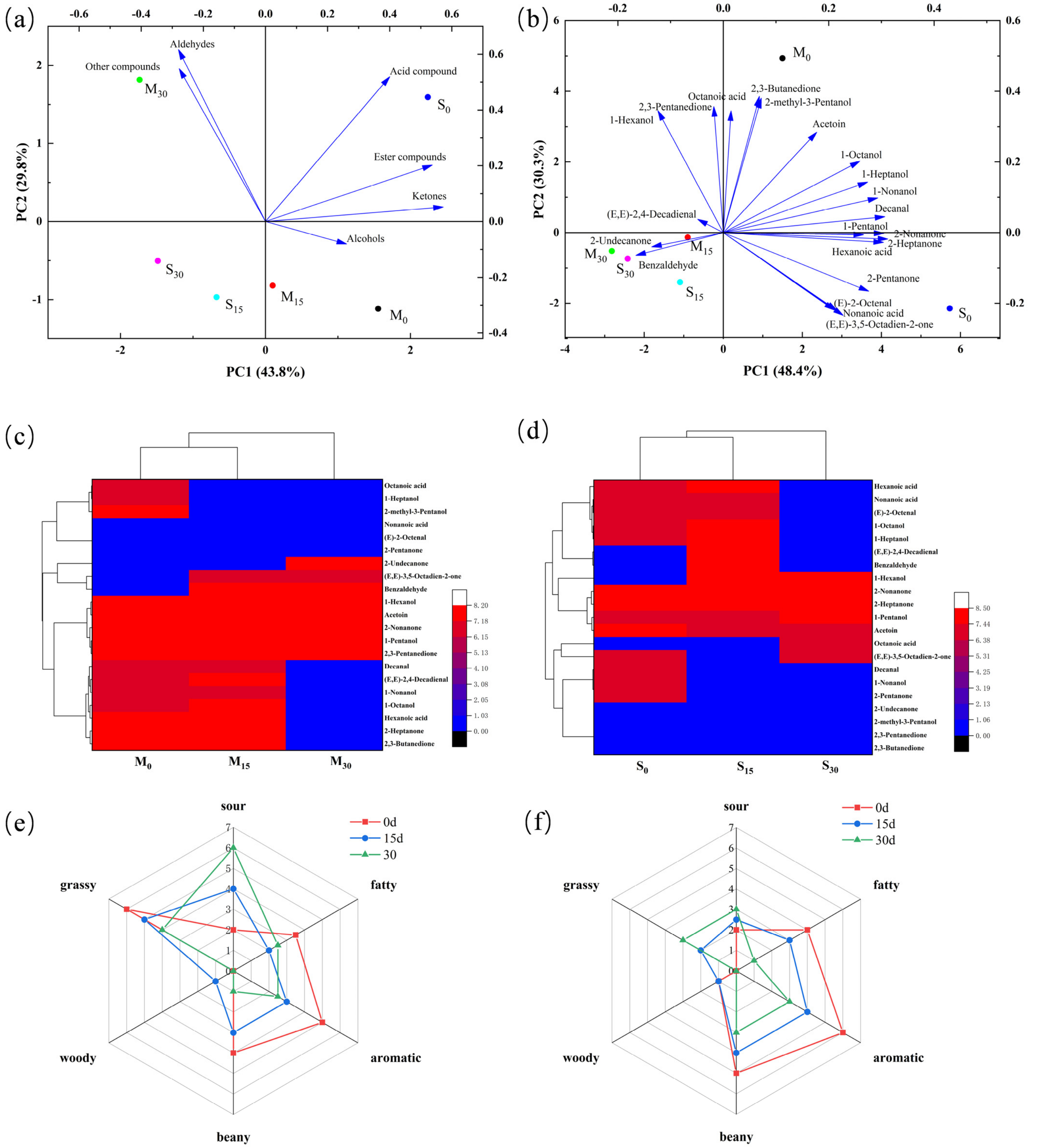

Figure 4. (**a**) Principal component analysis of volatile flavor substances during storage of the samples from the two mixed fermentation modes; (**b**) principal component analysis of the main volatile flavor substances during storage of the samples from the two mixed fermentation modes; (**c**) cluster analysis of the main volatile flavor substances during storage of the MFSM samples; (**d**) cluster analysis of the main volatile flavor substances during storage of the SMFSM samples (the volatile flavor substances were scaled according to the peak area using log10); (**e**) sensory scores of the MFSM samples during storage; and (**f**) sensory scores of the SMFSM samples during storage.

3.6. Textural Properties

The textural characteristics of fermented soymilk and milk mixtures from the two mixed fermentation methods were analyzed during storage (Table 2). The hardness, stickiness and chewiness of MFSM samples were significantly higher than those of the SMFSM samples throughout the storage period ($p < 0.5$). The MFSM samples are fermented with a soymilk and milk mixture directly to the end, and since casein and soy proteins aggregate simultaneously, they are more evenly dispersed throughout the network, giving the product greater firmness and chewiness [29]. Meanwhile, for the MFSM samples, milk is fermented alone first and then acidified and mixed with soymilk; thus, the product is in a semisolid form, so its hardness and chewiness are lower. The casein in the sample was first aggregated, and then the soy protein was integrated into the already preformed casein micelle network after the addition of soymilk. Therefore, the gel network may be less homogeneous, which has a greater impact on the texture of the sample [29]. The hardness and chewiness of the MFSM samples first increased and then decreased, while the stickiness did not significantly decrease. The cohesiveness also decreased significantly ($p < 0.5$) from 0 to 15 days and then did not change significantly ($p > 0.5$) but was greater than that at 15 days. The adhesion of SMFSM samples was significantly higher than that of MFSM samples during the storage period and did not change significantly ($p > 0.5$). These results indicate that different mixed fermentation methods have great effects on the texture characteristics of fermented soymilk and milk mixtures and on their stability during storage.

Table 2. Effects of different mixed fermentation methods on the texture characteristics of products.

	Time (d)	Hardness (N)	Adhesiveness (mJ)	Cohesiveness (Ratio)	Stickiness (N)	Chewiness (mJ)
	0	0.19 ± 0.00^{b}	0.14 ± 0.00^{d}	0.72 ± 0.02^{b}	0.13 ± 0.01^{a}	3.04 ± 0.10^{b}
MFSM	15	0.21 ± 0.01^{a}	0.17 ± 0.01^{d}	0.58 ± 0.01^{c}	0.11 ± 0.01^{a}	4.19 ± 0.02^{a}
	30	0.20 ± 0.00^{ab}	0.26 ± 0.03^{c}	0.57 ± 0.03^{c}	0.11 ± 0.01^{a}	4.08 ± 0.10^{a}
	0	0.07 ± 0.00^{c}	0.39 ± 0.03^{a}	0.86 ± 0.07^{a}	0.06 ± 0.01^{b}	1.77 ± 0.05^{c}
SMFSM	15	0.05 ± 0.00^{d}	0.33 ± 0.03^{b}	0.84 ± 0.06^{a}	0.04 ± 0.00^{c}	1.38 ± 0.09^{d}
	30	0.06 ± 0.00^{c}	0.26 ± 0.03^{c}	0.90 ± 0.07^{a}	0.06 ± 0.01^{b}	1.85 ± 0.10^{c}

$^{a–d}$ Mean values in same column with different lowercase superscripts differ significantly ($p < 0.5$).

3.7. Rheological Properties

The energy storage modulus (G') and loss modulus (G'') are important indicators of the rheological properties of fermented dairy products, mainly reflecting the magnitude of the gel strength. G' is related to the elasticity stored in the sample, and G'' is a fraction of the energy consumed by the sample during deformation [32]. The fermented soymilk and milk mixtures of both mixed fermentation methods showed an increasing trend of G' and G'' with increasing scan frequency throughout the storage period, as shown in Figure 5. G' and G'' of the MFSM samples were consistently higher than those of the SMFSM samples throughout the storage period, indicating a greater gel strength of the MFSM samples, probably due to the high acidity and low pH of the MFSM samples, which reduced the electrostatic repulsion between protein complexes in the samples and made the gel structure more stable [33], similarly to the previous results on the qualitative properties. G' and G'', respectively represent the degree of elasticity and viscosity of gel [34]. The samples of both mixed fermentation methods exhibited a significant increase in $G' > G''$, which is typical of weakly elastic gels and is consistent with the results of Haykuhi [35] et al., for both G' and G'' with increasing frequency during storage.

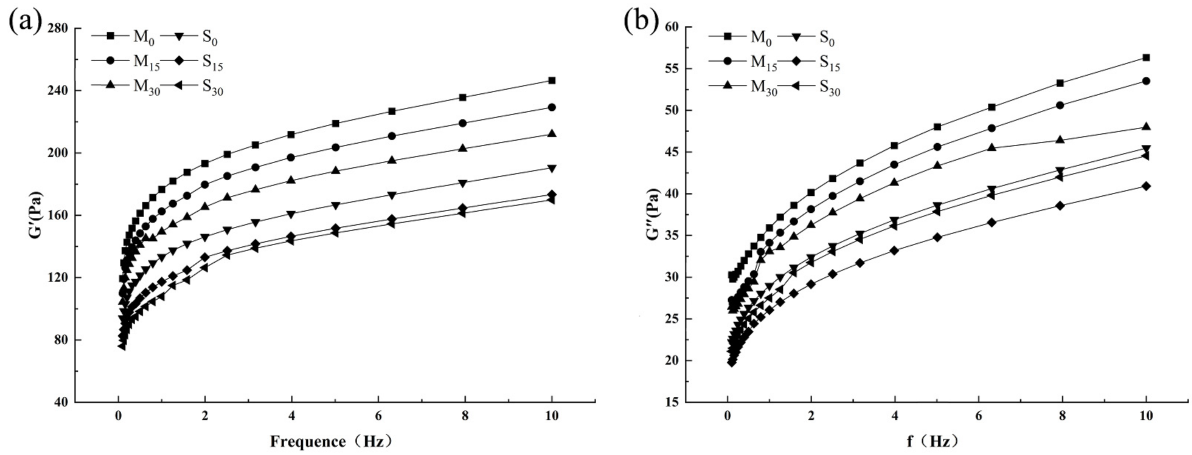

Figure 5. Frequency dependence of the storage modulus (G') and loss modulus (G''): (**a**) storage modulus (G') of the fermented soymilk and milk mixtures of the two mixed fermentation methods during storage and (**b**) loss modulus (G') of the fermented soymilk and milk mixtures of the two mixed fermentation methods during storage.

4. Conclusions

This study shows that as the acidity of soymilk and milk mixtures prepared by the two mixed fermentation methods during storage increases, the pH decreases and the number of viable bacteria can be maintained above 7 log cfu/mL, providing good probiotic properties. The number of *Lactobacillus bulgaricus subspecies* in the MFSM samples on the 0th day was lower than that of the SMFSM samples; however, the number of viable bacteria in the MFSM samples was higher than that of the SMFSM samples, and the lactic acid bacteria were still in the logarithmic growth period and vigorous at 70 °T in the MFSM samples. The MFSM samples were prepared in such a way that the plant and animal proteins were more tightly bound and the system was more stable, resulting in products with a higher water-holding capacity. However, the SMFSM method requires the fermentation of milk to a high acidity and then mixing with soymilk. The SMFSM method could significantly slow down the acidification of fermented soymilk and milk mixtures during storage so that the product exhibited good adhesiveness, and the water-holding capacity on the 30th day exhibited no significant change from that on the 0th day. The SMFSM samples had higher aromatic and soy flavors during storage than the fermented soymilk and milk mixtures in MFSM mode. Throughout the storage period, the content of 2-heptanone in the SMFSM samples was always higher than that of the MFSM samples, and on the 0th day, the content of 2-heptanone in the SMFSM samples was 2.1 times higher than that of the MFSM samples. On the 15th day, it was 1.47 times higher, and on the 30th day, no 2-heptanone was detected in the MFSM samples. The higher content of 2-heptanone can bring good flavor to the fermented products, which are well-liked by people.

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