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Effect of prebiotics on rheological properties and flavor characteristics of *Streptococcus thermophilus* fermented milk

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A R T I C L E I N F O

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A B S T R A C T

The fermentation characteristics and aroma production properties of lactic acid bacteria can influence the flavor quality of fermented milk, which is one of the important factors influencing the consumer preference. In this study, fermented milk was prepared using *Streptococcus thermophilus*, and dynamic changes in its quality, including rheological properties and flavor characteristics, were evaluated throughout the fermentation process. The results showed that benzaldehyde, 2-undecanone, octanoic acid, n-hexanol and 2-nonanol were the key flavor components during the fermentation process. The quality of the fermented milk tends to be stabilized after 24-h, showing the minimal off-flavor and optimal fermented aroma at 48-h. Three prebiotics (inulin, Galactooligosaccharides and inulin mixed with Galactooligosaccharides) were added to *Streptococcus thermophilus* fermented milk separately, and the results showed that inulin was the most effective group in improving the organoleptic quality of the fermented milk. These findings contribute to our understanding of the release and retention of flavor compounds during fermentation and can be used as a scientific reference for the application of probiotics and flavor-producing lactic acid bacteria in fermented milk processing.

1. Introduction

Fermented dairy products represent a unique category of nutritious and beneficial acidic curd-like products through the fermentation process facilitated by lactic acid bacteria (LAB) (X. Yu et al., 2024). LAB carrying out the fermentation produce lactic acid from lactose, so increasing the acidity of the products, and they have a highly complex peptidase system, which is usually important in the fermentation of dairy products. Thus far, LAB fermentation significantly alters the composition of metabolites such as organic acids, free fatty acids and other flavor metabolites have been reported in numerous studies (Galli et al., 2022). Certain fermenters can help improve the texture and flavor profile of fermented products (Cheng et al., 2024). Sensory texture characteristics are important indicators of yogurt quality and drivers of consumer choice. The flavor of fermented dairy products is influenced by many factors such as dairy source, processing parameters, chemicals, fermenters and food additives (Wu et al., 2023). Among these factors, fermenters are important to the formation of flavor compounds (Tian et al., 2019). *Streptococcus thermophilus* rapidly produces acid, metabolizes lactose, and produces extracellular polysaccharides, vitamins, and flavor substances during dairy production. It is generally considered a safe strain and, thus is widely used as a fermenter in commercial yogurt production (T. Wu et al., 2023). High-quality fermented milk usually has excellent texture, stability and flavor. In practice, *Streptococcus thermophilus* used in the production of fermented milk, as the main bacterium responsible for the flavor, can significantly influence the sensory quality of yogurt (Dan et al., 2023; Iyer et al., 2010; Nsogning Dongmo, Procopio, Sacher and Becker, 2016; C. Xu et al., 2017).

Functional dairy products with potential health benefits (e.g., probiotic yogurt) have seen rapid growth in recent years due to the growing interest in healthy eating of the population (Tian et al., 2017). Prebiotics are substrates that are selectively utilized by host microorganisms conferring health benefits (Gibson et al., 2017). Prebiotics could not only control the texture and rheology of fermented milk but also create a symbiotic fermented dairy product to meet the multidimensional needs of consumers (D. Yu et al., 2021). Inulin can be extracted from a variety of sources including chicory, garlic, wheat, oats, and dahlia (Bedani et al., 2013). It is an oligosaccharide (fructan) with a β-2-1-linked fructose unit and a terminal glucosyl unit that can be used as a prebiotic by LAB and can promote the proliferation of beneficial bacteria in the gastrointestinal tract, in addition to improving the texture, chemical, functional and sensory properties of fermented milk (Canbulat and

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Ozcan, 2015; Cardarelli et al., 2008; Crispín-Isidro et al., 2015; Fang et al., 2023; Guven et al., 2005; Hekmat and Reid, 2006; Kamel et al., 2021). Galacto-oligosaccharides (GOS) are indigestible oligosaccharides with prebiotic properties (Belsito et al., 2017; Lamsal, 2012; López-Sanz, Moreno, de la Mata, Moreno and Villamiel, 2018). GOS has been shown to affect the gut microbiota and have barrier function and other beneficial health effects in humans and animals, including improved feces, mineral absorption, weight management, carcinogenesis and allergy relief (Lamsal, 2012; G. T. Macfarlane et al., 2007; G.T. Macfarlane et al., 2008). GOS is commonly added as a beneficial ingredient in infant formula, in addition to improving the texture of fermented milk due to its excellent solubility (El-Ghaish et al., 2011).

Various analytical methods have been used in the last decades to study the flavor of fermented milk. Artificial sensory evaluation is the most important and effective method. However, it is time-consuming and can be influenced by the physiological and psychological state of the taster. Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) is an efficient and accurate method for the non-targeted determination of complex flavor metabolism components in fermented milk; it has been used to assist in the sensory evaluation of dairy products (Tian et al., 2017). Consumers expect fermented dairy products to have unique textures and flavors (Sakandar and Zhang, 2021). In addition to its aroma profile, the rheological properties of fermented milk are crucial parameters that should also be considered (Buldo et al., 2021). Dynamic changes in rheological properties and flavor characteristics can occur during fermentation using *Streptococcus thermophilus* as a fermenter. With the addition of prebiotics, they decisively change their organoleptic texture characteristics and the release of some flavor compounds. Thus, to produce new fermented foods with better quality, it is worth exploring the effect of prebiotics on the flavor profile during fermentation.

The aims of this study are to investigate the promotional effects of prebiotics on fermentation characteristics and flavor release during fermentation by multivariate data analysis methods and to apply physicochemical, sensory evaluation and untargeted flavoromics techniques to assess the influences of prebiotics on the apparent changes in qualities and intrinsic metabolites of *Streptococcus thermophilus* fermented milk. The findings can provide new insights into the mechanism of flavor formation and quality regulation in fermented milk.

2. Materials and methods

2.1. Materials

Streptococcus thermophilus GST-6 was isolated from Tibetan kefir grain and preserved in a dairy laboratory at Beijing Technology and Business University, Beijing, China. Whole milk powder was purchased from Fonterra Co-operative Group Ltd. (Auckland, New Zealand). Brain heart infusion broth (BHI) medium was obtained from AOBOX Co., Ltd. (Beijing, China). Prebiotics including inulin and GOS were purchased from LuAn Biotechnology Limited Co., Ltd. (Shanghai, China). Sodium chloride (AR) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Butanol and n-alkanes (C7 – C40) were purchased from J&K Scientific Limited Co., Ltd. (Beijing, China).

2.2. Sample preparation

BHI medium was sterilized at 121 °C for 15 min. *Streptococcus thermophilus* GST-6, stored at −80 °C, was cultured in BHI medium at 42 °C for 18 h. After two successive subcultures, the strain was stored at 4 °C.

The fermented milk was prepared based on the National Standard of the People's Republic of China (2010; standard GB, 19302–2010) and a previously published work (Yang et al., 2021) with modifications. The whole milk powder was reconstituted to 8 % (w/v) total solids in the water at 25 °C, and subsequently these mixtures were mechanically agitated using laboratory disperser to obtain uniform distribution of sucrose (5%) in the whole milk powder/water blend. The samples were pasteurized at 65 °C for 30 min and cooled to the incubation temperature (37 °C). *Streptococcus thermophilus* was added to the milk at 2% (V/V) to produce fermented milk. Sucrose was added as a sweetener. Sucrose was added as a sweetener and milk was fermented at 42 °C. Subsequently, samples were collected at 3 h, 6 h, 12 h, 24 h, 48 h and 72 h and stirred for 10 min before further GC-MS analysis and sensory evaluation. Rheological parameters and pH were measured at 1h intervals during fermentation.

For the control group, 3% (w/v) of inulin (Inulin group), 3% (w/v) of GOS (GOS group), and 3% (w/v) of the mixture (inulin and GOS at a ratio of 1:1 (Inulin/GOS group)) were added to the reconstituted milk, and other treatments remained the same.

2.3. Rheological properties

Microrheological parameters of fermented milk, including elasticity index (EI), melt viscosity index (MVI), fluidity index (FI) and solid-liquid balance (SLB), were determined by the method reported in Bai et al. (2020). Fermented milk sample (20 mL) was added to vials in a micro rheometer (LAB 6 MASTER, Formulaction Inc., Toulouse, France), and the microrheological parameters during the gel formation process at 42 °C were monitored.

Changes in pH during fermentation were determined using an iCinac dairy fermentation monitor system (AMS-Alliance, France).

2.4. Flavor characteristics of fermented milk

2.4.1. Extraction of flavor compounds

Fermented milk (8 mL), butanol (10 μL), and sodium chloride (1 g) were added to a 20-mL headspace bottle. After being incubated in headspace vials at 60 °C for 15 min, volatiles were extracted through a DVB/CAR/PDMS fiber (50/30 μm, 1 cm, Supelco, Bellefonte, PA, USA) for 20 min. Then, the fiber was inserted into a GC injector to allow the desorption at 250 °C for 5 min.

2.4.2. GC-MS analysis

The volatiles of fermented milk were analyzed by 7890-5975 gas chromatograph-mass spectrometry using a polyethylene glycol capillary column DB-WAX (30 m × 0.25 mm, 0.25 μm film thickness, Agilent, USA).

The oven temperature was set at 40 °C for 3 min and then heated up at 5 °C/min to 140 °C for 2 min After that, it was being ramped up to 240 °C at 10 °C/min and held for 1 min. The mass spectrometer was operated in full scan mode. Data acquisition was performed in the m/z range of 35–500.

2.4.3. Identification and quantitation of the volatile compounds

Semi-quantification of volatiles was based on the linear relationship between the peak area and the concentrations of internal standard (butanol, 2.75 mg/L) and volatile compounds. Tentative identifications were carried out by matching the mass spectra of the unknowns with those in the NIST 2014 database (NIST/EPA/NIH Mass Spectral Library, ver. 2.3, 2014) at a threshold of an ion-matching score greater than 80%.

2.4.4. Odor activity value (OAV)

OAV analyses were performed to determine the contribution of each component to the overall flavor. The calculation was carried out by the following formula (H. Xu et al., 2024):

$$OAV = \frac{C}{T}$$

Where C and T represent the concentration and order threshold of the target compound.

2.5. Sensory evaluations

The quantitative descriptive analysis (QDA) was conducted in a sensory evaluation laboratory. All fermented milk samples were assessed by six well-trained panelists from the School of Food and Health, Beijing Technology and Business University. In the rating of the odor intensity of each sample, a 0 to 5 score method was used by which different odor attributes were given a score (Table S1).

All participants have given an informed consent statement before participating in the study. Ethical approval for the involvement of human subjects in this study was granted by the Beijing Technology and Business University Research Ethics Committee (Reference number 44, 2024).

2.6. Statistical analysis

All experiments were conducted in triplicate, and the results were presented as mean ± standard error of the mean. The correlation heat map, Venn diagram and network diagram were performed at https://www.chiplot. The principal components analysis (PCA) was performed by SIMCA14.1 (Sartorius Stedim Data Analytics AB, Umea, Sweden). Radar chart and response curve were prepared using Origin Pro 2022 (Origin Lab, Northampton, MA, USA), and the graphs were generated using GraphPad Prism v9.0.1 (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Fermentation characteristics

Microrheology is a technique to determine the viscoelastic properties of systems by measuring the Brownian motion of particles in the absence of mechanical or external force. Rheological properties are the basic indicators of fluid stability and the basis for fermented milk processing and quality control (J. Wu et al., 2023). Dynamic changes of microrheological parameters, such as EI, FI, MVI, and SLB, were monitored during fermentation (Fig. 1A–D).

The texture of fermented milk has a certain viscoelasticity which depends on rheological properties such as solid-liquid transformation. The increase of EI with acidification is caused by particle aggregation and gel network formation (J. Wu et al., 2023). The EI values were related to the formation of structures. This value did not change significantly at the beginning but increased rapidly after 11 h, and the trend continued as the fermentation time was extended, which suggests that the interactions among the droplets of the emulsions were enhanced.

The MVI value represents the fluidity of droplets in a fermented milk system (D. Xu et al., 2016). As the EI values changed, fermented milk had a low viscosity initially and appeared to be relatively unstable (Fig. 2B). This shows that the droplet elasticity and viscosity have a positive effect on the emulsion stability (T. Wu et al., 2023). The MVI value increased rapidly after 16 h and continued to increase with the acidification process until being stabilized at a relatively high level. This indicates that the samples entered the high viscosity stage, and the gel structure became more stable.

FI demonstrates the speed of movement of microscopic particles of fermented milk, and the mobility of droplets in the system is usually negatively correlated with MVI. The FI value decreased rapidly from 124 to 1.05 after 12 h; after that, the decreasing rate slowed down until reaching a stable value after 29 h. The value finally stayed at a low level as the fermentation time was extended. As the viscosity increased, the mobility of the emulsion decreased.

The SLB value during fermentation peaked at 0.76 after 10 h but was in an unstable stage during the pre-fermentation period. Finally, the value stabilized at 0.58 after 43 h. Microrheological tests showed that the fermented milk had inherent solid characteristics at about 40 h of fermentation.

The acidity development was monitored during the fermentation process to track acidification of fermented milk samples as a key factor affecting the organoleptic properties, microbial activity and quality of the fermented (D. Xu et al., 2016). At the beginning of fermentation (at about 3 h), the pH value was greater than 6.0. This value decreased slowly as the acidizing time was further extended. Then, it rapidly decreased to 5.0 until finally stabilized at 4.5 after 46 h (Fig. 1E). During the fermentation process, *Streptococcus thermophilus* continued to produce acid, the number of microorganisms increased, the accumulated organic acid content increased, and the low acidity environment promoted fermentation. The pH value of fermented milk is closely correlated with the growth and metabolism of bacteria during the acidification process. When the pH is reduced to a certain level, the growth and metabolism of LAB are inhibited and eventually reach equilibrium (Crispín-Isidro et al., 2015). The acid-producing ability of the strains and the accumulation of organic acids can dynamically change of sweet/sour properties of fermented milk.

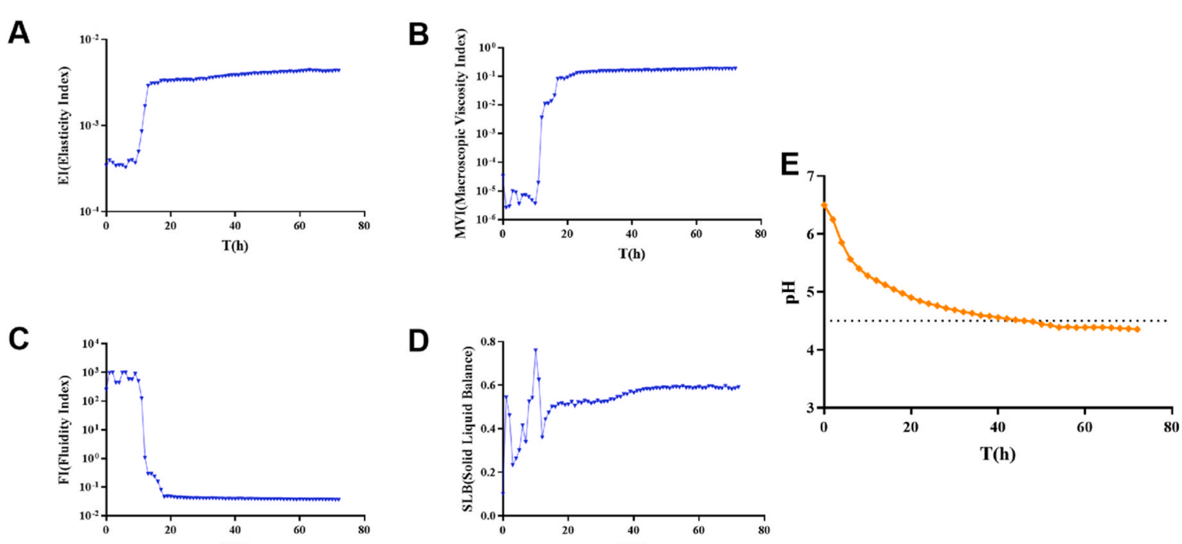

Fig. 1. Microrheological properties and pH of fermented milk during fermentation progress. Elasticity index **(A)**; Macroscopic Viscosity Index **(B)**; Fluidity Index **(C)**; Solid-Liquid Balance **(D)**; and pH **(E)**.

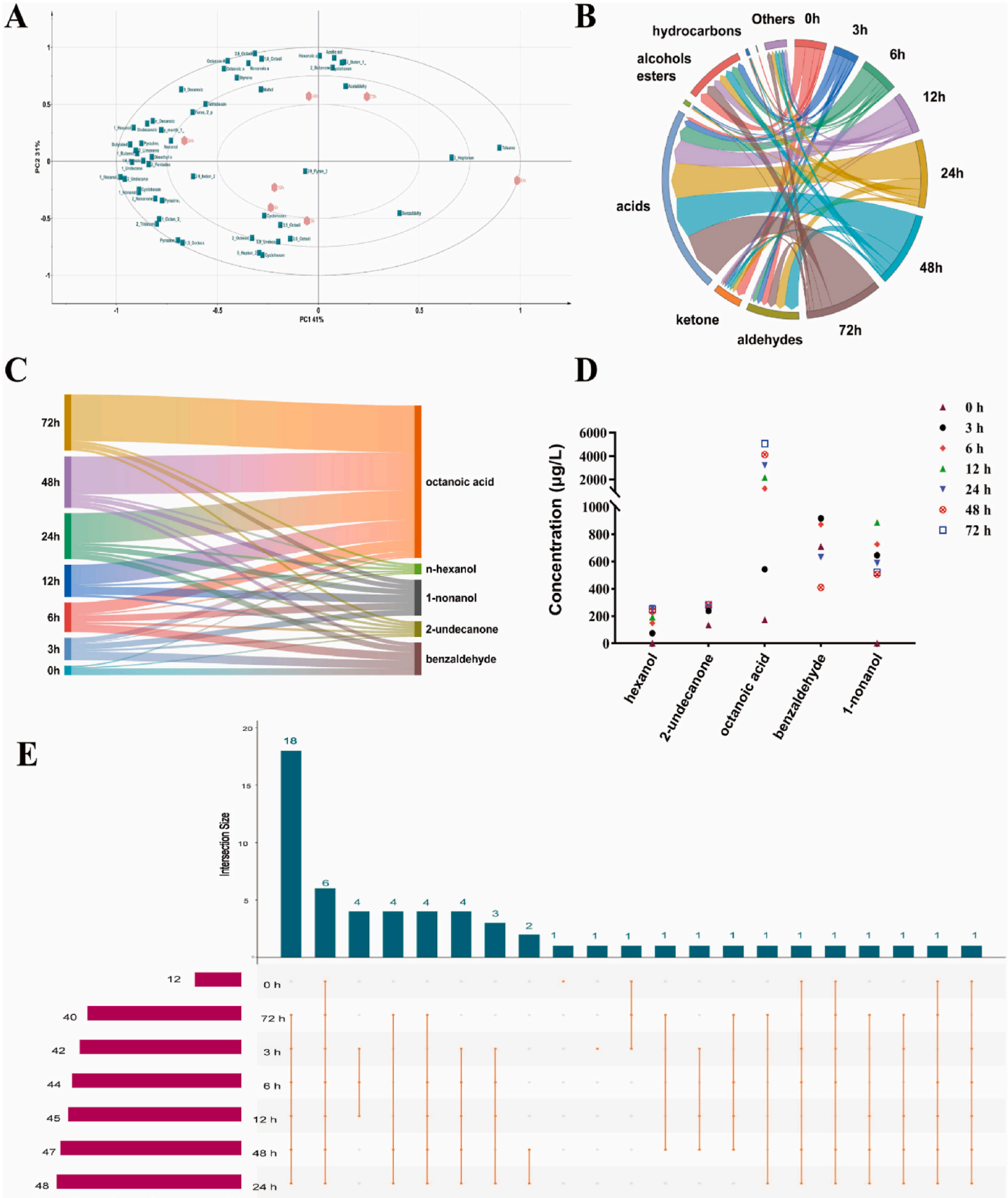

Fig. 2. PCA score scatter plot **(A)**; The categories of the volatiles **(B)**; Dynamics of key flavor compounds during fermentation **(C)**; The concentration of key aroma-active compounds **(D)**; Upset plot of volatile compounds during fermentation **(E)**.

Combining all indicators, including rheological parameters and pH, the results showed that fermented milk prepared using *Streptococcus thermophilus* as the fermenting agent reached the stabilization stage after 40 h. Therefore, the analysis of flavor release and sensory quality evaluation of fermented milk should be carried out from 40 to 72 h.

3.2. The performance of flavor in fermented milk

3.2.1. Compounds identified by GC-MS

By employing HS-SPME/GC-MS, 57 volatile components, including 13 alcohols, 9 aldehydes, 10 ketones, alkenes, 4 esters, 8 acids, 3 hydrocarbons, and 10 others, were identified during fermentation. The detailed information on volatile organic compounds is presented in Table 1. The samples had not started to ferment at 0h, and compared to fermented milk, the recovered milk powder contained fewer volatile compounds, mainly aldehydes and ketones, and no acidic compounds were detected. The results from the principal component analysis (Fig. 2A) showed that the flavors of samples slightly changed during the first 12 h of fermentation, and their distribution distances could easily be separated. Even more obvious is the gap between fermented milk and the initial phase of reconstituted milk powder. By contrast, after 24 h, the volatiles changed significantly. The dynamics of the composition

Table 1
The results of flavor substances during fermentation of *Streptococcus thermophilus*.

RT	Compound	CAS	RI	RI_{ref}	Concentration (μg/L)						
					0 h	3 h	6 h	12 h	24 h	48 h	72 h
2.04	acetaldehyde	75-07-0	656	713	–	–	–	–	1891.94 ± 72.48	2296.78 ± 193.93	–
4.6	diacetyl	431-03-8	960	965	–	–	–	–	250.66 ± 28.56	254.65 ± 14.60	–
5.86	toluene	108-88-3	1034	1036	33.30 ± 2.86	0.78 ± 0.07	–	–	–	–	1.21 ± 0.33
6.9	hexanal	66-25-1	1076	1077	214.63 ± 115.61	145.22 ± 16.78	5.95 ± 0.20	24.02 ± 2.72	–	–	–
9.1	isobutyl butyrate	539-90-2	1157	1160	–	5.49 ± 1.92	0.61 ± 0.16	1.23 ± 0.12	–	–	–
9.69	2-heptanone	110-43-0	1178	1184	515.93 ± 48.60	293.49 ± 38.37	280.08 ± 26.50	–	225.57 ± 33.83	146.13 ± 22.58	71.40 ± 1.73
9.98	3-methyl-2-butenal	107-86-8	1188	NF	–	–	–	–	305.86 ± 21.17	787.97 ± 134.31	1156.44 ± 134.52
10.03	dipentene	138-86-3	1190	1185	–	69.69 ± 4.01	114.68 ± 9.26	94.00 ± 10.02	–	–	–
10.42	isopentyl alcohol	123-51-3	1204	1185	–	–	–	–	14.51 ± 0.57	–	46.92 ± 1.83
11.04	2-pentylfuran	3777-69-3	1226	1249	–	67.60 ± 12.16	58.54 ± 11.51	68.75 ± 15.85	118.43 ± 28.29	92.62 ± 6.86	152.27 ± 15.8
11.69	1-Pentanol	071-41-0	1250	1255	75.50 ± 24.92	–	–	–	–	–	–
11.88	2-methylpyrazine	109-08-0	1257	1265	45.28 ± 6.13	51.97 ± 8.10	42.82 ± 4.82	59.29 ± 6.78	61.41 ± 10.93	64.72 ± 12.01	69.75 ± 15.83
12.36	ethyl diphosgene	513-86-0	1274	1271	–	–	72.57 ± 12.26	134.06 ± 26.00	214.16 ± 17.69	151.18 ± 1.54	274.89 ± 44.94
12.55	octanal	124-13-0	1281	1286	–	90.90 ± 22.25	–	39.95 ± 4.83	–	58.58 ± 19.44	–
13.42	2,5-dimethylpyrazine	123-32-0	1313	1321	–	604.11 ± 109.11	621.57 ± 79.78	558.58 ± 63.71	641.46 ± 107.82	517.27 ± 48.04	535.35 ± 34.18
13.61	2,6-dimethylpyrazine	108-50-9	1320	1330	–	20.91 ± 3.01	26.41 ± 3.21	24.14 ± 3.83	24.92 ± 5.33	22.10 ± 2.33	16.45 ± 0.21
13.79	methylheptenone	110-93-0	1327	1323	–	–	1.49 ± 0.21	1.24 ± 0.21	–	21.04 ± 5.37	80.99 ± 11.64
14.36	n-hexanol	111-27-3	1348	1360	–	74.56 ± 3.61	148.08 ± 16.68	190.96 ± 22.68	259.84 ± 14.54	236.98 ± 43.47	248.40 ± 42.32
15.19	2-nonanone	821-55-6	1379	1387	–	312.53 ± 51.42	279.62 ± 36.90	191.74 ± 14.81	157.06 ± 12.71	83.11 ± 6.19	–
15.29	nonanal	124-19-6	1383	1395	–	372.90 ± 38.41	367.43 ± 32.75	301.12 ± 47.01	394.41 ± 70.01	816.56 ± 115.4	1470.30 ± 576.71
15.47	tetradecane	629-59-4	1390	1400	55.60 ± 17.49	39.32 ± 7.08	48.98 ± 7.97	24.68 ± 5.13	57.92 ± 13.61	84.70 ± 18.67	201.87 ± 55.46
15.62	2,3,5-trimethylpyrazine	14667-55-1	1395	1402	–	88.56 ± 14.65	71.32 ± 12.66	73.25 ± 9.48	82.26 ± 12	64.58 ± 6.77	75.85 ± 9.63
16.18	trans-2-octenal	2548-87-0	1417	1434	–	34.50 ± 1.25	38.59 ± 2.85	18.58 ± 4.86	5.25 ± 0.35	–	–
16.39	ethyl caprylate	106-32-1	1425	1420	–	–	–	–	12.24 ± 0.03	16.76 ± 3.68	30.70 ± 7.06
16.67	3-ethyl-2,5-methylpyrazine	13360-65-1	1436	1430	–	136.32 ± 9.40	135.80 ± 21.19	108.40 ± 26.32	158.20 ± 13.33	166.22 ± 43.59	311.03 ± 3.20
16.86	1-octen-3-ol	3391-86-4	1444	1430	–	194.40 ± 21.91	174.74 ± 18.56	146.86 ± 16.94	161.01 ± 12.43	102.31 ± 12.12	175.21 ± 9.57
17	heptyl formate	112-23-2	1449	NF	–	175.00 ± 25.26	239.27 ± 28.39	231.09 ± 33.78	254.12 ± 9.20	204.51 ± 3.63	–
17.32	acetic acid	64-19-7	1462	1433	–	–	–	0.71 ± 0.20	154.89 ± 24.22	329.86 ± 12.95	191.40 ± 16.89
17.86	2-ethylhexanol	104-76-7	1483	1480	433.23 ± 68.76	412.97 ± 29.5	487.35 ± 93.52	481.65 ± 80.37	504.07 ± 24.35	748.32 ± 107.77	1402.71 ± 104.09
18.47	benzaldehyde	100-52-7	1507	1520	475.09 ± 52.54	915.68 ± 127.01	871.25 ± 112.55	–	631.71 ± 71.06	409.46 ± 23.25	–
18.58	2-nonanol	628-99-9	1512	1530	–	–	4.87 ± 0.60	18.27 ± 3.78	29.88 ± 3.74	25.24 ± 3.21	30.23 ± 6.30
19.23	linalool	78-70-6	1539	1554	–	27.93 ± 2.74	48.56 ± 3.54	46.78 ± 5.25	91.49 ± 4.52	115.79 ± 10.19	165.11 ± 14.30
19.52	octanol	111-87-5	1551	1558	–	362.19 ± 57.02	457.94 ± 62.88	415.91 ± 40.40	455.98 ± 52.52	362.07 ± 34.29	411.71 ± 51.42
19.6	3-methylpentadecane	2882-96-4	1554	NF	–	–	–	–	–	39.82 ± 2.76	100.41 ± 5.04
19.67	3,5-octadien-2-one	30086-02-3	1557	1516	–	35.45 ± 7.31	33.88 ± 5.97	27.32 ± 0.19	22.38 ± 3.20	27.71 ± 2.06	–
20.39	2-undecanone	112-12-9	1587	1599	–	238.65 ± 15.14	248.50 ± 23.93	246.36 ± 33.80	265.90 ± 40.04	282.28 ± 24.18	281.37 ± 4.31

(*continued on next page*)

Table 1 (*continued*)

RT	Compound	CAS	RI	RI_{ref}	Concentration (μg/L)						
					0 h	3 h	6 h	12 h	24 h	48 h	72 h
20.58	tea pyrrole	2167-14-8	1595	1616	–	20.68 ± 3.57	35.78 ± 4.32	21.24 ± 2.24b	22.66 ± 3.91	12.61 ± 2.42	15.30 ± 3.90
21.32	phenylacetaldehyde	122-78-1	1626	1636	243.96 ± 9.10	235.62 ± 0.79	238.17 ± 3.94	48.25 ± 0.68	40.54 ± 6.40	35.21 ± 6.48	–
21.52	butyric acid	107-92-6	1635	1628	–	–	–	–	327.58 ± 12.3	264.27 ± 57.98	1397.71 ± 47.52
21.91	1-nonanol	143-08-8	1652	1640	–	644.80 ± 27.63	726.95 ± 114.96	884.63 ± 102.24	587.46 ± 76.91	506.48 ± 39.72	521.35 ± 56.08
23.26	2-undecanol	1653-30-1	1709	1706	–	–	0.81 ± 0.15	3.23 ± 0.17	21.06 ± 2.63	17.59 ± 2.27	21.36 ± 5.91
25.52	2-tridecanone	593-08-8	1797	1814	–	58.58 ± 2.77	68.96 ± 10.53	39.97 ± 8.57	36.65 ± 3.22	25.45 ± 4.06	33.97 ± 5.83
26.46	geraniol	106-24-1	1841	1841	–	–	–	10.39 ± 1.80	53.74 ± 8.99	56.19 ± 5.67	69.22 ± 4.11
26.54	geranylacetone	3796-70-1	1845	1856	–	15.44 ± 3.27	18.01 ± 0.87	17.92 ± 0.56	–	–	–
26.84	hexanoic acid	142-62-1	1859	1827	–	124.22 ± 1.10	1012.53 ± 135.31	2104.83 ± 265.01	2298.41 ± 191.7	2842.93 ± 293.84	3087.3 ± 582.39
27.33	dimethyl sulfone	67-71-0	1883	1912	–	22.10 ± 5.60	18.49 ± 3.48	20.09 ± 4.42	65.09 ± 5.77	60.78 ± 13.18	39.05 ± 3.70
27.65	butylhydroxytoluene	128-37-0	1899	1911	–	19.32 ± 4.75	31.82 ± 7.65	31.87 ± 4.54	51.25 ± 4.69	72.02 ± 12.58	104.96 ± 16.55
27.87	2-tridecanol	1653-31-2	1912	NF	–	–	–	–	9.26 ± 2.39	8.05 ± 1.31	13.66 ± 1.84
28.63	dodecanol	112-53-8	1960	1983	–	57.21 ± 1.40	88.67 ± 7.76	77.65 ± 13.06	110.10 ± 21.95	114.11 ± 11.51	114.01 ± 27.30
29.36	2-pentadecanone	2345-28-0	2008	2021	–	25.66 ± 0.43	22.84 ± 4.51	13.26 ± 0.42	8.00 ± 0.65	–	–
29.47	pentadecanal	2765/11/9	2016	2054	–	6.07 ± 0.03	–	–	–	–	–
30.27	octanoic acid	124-07-2	2077	2050	–	541.85 ± 95.09	1223.30 ± 25.95	2145.14 ± 316.80	3215.95 ± 131.83	4118.22 ± 309.28	5067.68 ± 1152.64
31.56	butyldecalactone	705-86-2	2187	2203	137.36 ± 24.29	18.24 ± 0.34	20.38 ± 5.77	15.62 ± 1.29	27.61 ± 0.76	–	–
31.66	nonanoic acid	112-05-0	2196	2144	–	–	89.09 ± 14.69	–	136.30 ± 5.81	143.84 ± 29.95	241.86± 26.65
32.7	decanoic acid	334-48-5	2297	2279	–	900.49 ± 144.67	1575.88 ± 125.15	1961.59 ± 132.70	3383.33 ± 471.54	2958.36 ± 379.61	3846.47 ± 565.29
33.38	9-decenoic acid	14436-32-9	2368	2335	–	–	290.77 ± 56.23	444.66 ± 33.02	724.47 ± 55.88	626.59 ± 9.72	455.36 ± 9.96
34.74	lauric acid	143-07-7	2520	2508	–	397.34 ± 81.06	705.61 ± 5.26	616.78 ± 111.21	1064.58 ± 200.88	1380.52 ± 91.48	1065.29 ± 94.87

Note: Results are presented as the mean ± standard deviation.
RI (retention index) was calculated by analyzing a C7 – C40 alkane mixture under the above GC - MS condition.
RI_{ref} (retention index reference) referred from web: https://webbook.nist.gov/chemistry/name-ser/.
—, means not detected.

and content of volatiles reflect the growth and metabolic state of *Streptococcus thermophilus* during fermentation (Fig. 2B). The contribution of flavor-active compounds depends not only on the concentration but also on the threshold value (Tian et al., 2020; H. Xu et al., 2024). According to the principle of OAVs, octanoic, n-hexanol, 2-undecanone, 1-nonanol and benzaldehyde were considered as the contributing components to the flavor of fermented milk (Fig. 2C). Other volatiles had an OAV value of 1, which is in agreement with the overall flavor of fermented milk. Fig. 2D illustrates the specific concentrations of key active flavor compounds during fermentation. The OAV values of other volatiles were below 1, which meant that they would play a supplementary role in the overall flavor of fermented milk (Tian et al., 2017).

Aldehydes are common volatile compounds in fermented milk and act as active flavor compounds due to their low thresholds. As the acidification was enhanced, many aldehydes that were detected earlier disappeared while some new compounds were subsequently produced. Aldehydes detected during the initial stage of fermentation may be derived from the samples, the heat treatment of the recovered milk, or the metabolism during the rapid proliferation phase of LAB. Aldehydes can be easily reduced to alcohols or oxidized to acids (Sun et al., 2023). Among all the flavor compounds, acetaldehyde and diacetyl are considered the characteristic compounds and are important indicators for assessing the quality of fermented milk (Tian et al., 2019). The acetaldehyde content, which is the main compound that provides fermented milk a typical aroma (green, nutty), increased cumulatively throughout the fermentation process. The contents of butenal and acetaldehyde increased with increasing fermentation time and peaked at 48 h. The content of pentadecanal was only detected during the initial stage of fermentation (3 h). The concentration of benzaldehyde decreased over time and was below the detectable level after 72 h of fermentation. During the pre-fermentation stage, some reactions may occur, and the Strecker degradation of phenylalanine in fermented milk may take place, forming a large amount of benzaldehyde. It is likely that, as the pH value decreased during fermentation and the LAB continued to proliferate, benzaldehyde was gradually degraded during acidification and converted into other substances such as phenol and benzoic acid.

Ketones are derived from the oxidative and thermal degradation of unsaturated fatty acids, the metabolism of amino acids, and the metabolism of LAB (Tian et al., 2020). A total of 10 ketones were detected during fermentation. Among all the ketones, diacetyl, which has a creamy flavor and is derived mainly from citric acid metabolism, has been repeatedly detected as the main flavor substance in fermented milk (Fang et al., 2023; Guven et al., 2005). Throughout the fermentation process, diacetyl was present in all fermented milk except for that at the

72-h time point. Its concentration increased with fermentation time, peaking at 48 h. The contents of 2-heptanone, 2-nonone and 2-tridecone, which are common metabolites in yogurt and are the major contributors to sweet, fruity and milky flavors, also increased with fermentation time. In contrast, the contents of 2-undecone (floral and herbal) gradually decreased during fermentation. Ethyl diphosgene, a reduced form of diacetyl with a creamy flavor, was present at a much lower level than diacetyl (Guven et al., 2005). The concentration of diacetyl tended to increase with fermentation time, and ethyl diphosgene was detected earlier than diacetyl, which is an indication that the production of ethyl diphosgene occurs before diacetyl (Bai et al., 2020). The flavor of fermented milk is the result of the overall combination of volatile metabolites. Increasing the concentration of key volatiles can improve the sensory quality of fermented milk.

Esters are produced via the esterification reaction between alcohols and carboxylic acids or amino acids. In general, most esters have floral, fruity, and wine aromas that can reduce the bitterness and other odors produced by fatty acids and amines (Güler, 2007; T. Wu et al., 2023). The contents of ethyl caprylate increased with increasing fermentation time. As the fermentation progressed, the content of heptyl formats first increased until reaching its peak value at 24 h and then decreased. Ethyl caprylate has been reported to be an odor-active volatile in fermented brown milk, imparting a sweet taste to the milk (Wang et al., 2024; T. Wu et al., 2023).

During the fermentation process, it could be observed that the acetic acid content peaked after 48 h, while the hexanoic acid and octanoic acid contents increased gradually. Organic acids are natural preservatives that affect various aspects of the sensory and structure of fermented dairy products. The main organic acid produced during lactic acid bacteria fermentation is lactic acid (El-Ghaish et al., 2011). Other organic acids are also produced in fermented milk due to the homofermentative metabolic activity of lactic acid bacteria. These compounds have been reported to have some synergistic olfactory effects (Tian et al., 2019; T. Wu et al., 2023). The decanoic acid content first increased and then decreased during fermentation, and the value was lowest in the samples fermented for 48 h. Decanoic acid is a fatty acid that tends to introduce off-flavors into samples with high fat content (Tian et al., 2020). The content of lauric acid fluctuated during fermentation and did not show a clear linear relationship; however, the value peaked at 48 h and then continued to decrease. This compound contributes to the nutty and woody aroma of fermented milk, making its overall flavor richer and more harmonious. Acidic compounds were not detected in the reconstituted milk powder samples, and as the fermentation time increased, the type and content of acidic compounds gradually increased, the bacteria began to function, and the samples began to undergo qualitative changes.

Alcohols are mainly derived from fatty acid reduction, methyl ketone oxidation, amino acid degradation and sugar metabolism (Tian et al., 2020). Alcohols were the most diverse substances in all samples, and despite their high thresholds, they still had a significant impact on the flavor. During fermentation, the n-hexanol content varied and reached its maximum value at 48 h. 1-Octen-3-ol (mushroom alcohol) is a typical source of fishy, mushroom flavor. High concentrations of mushroom alcohol have been repeatedly reported to be an off-flavor compound in dairy products. At the lowest concentration and after 48 h, this volatile compound could result in the weakest off-flavor, in turn affecting the overall preference. 2-Ethylhexanol concentration decreased with fermentation time, while linalool concentration increased with increasing time throughout the fermentation. Dynamic changes in their concentrations directly affect the aroma attribute scores of the fermentation.

Hydrocarbons, mainly from fat, sugar and amino acid metabolism, were present in the samples at the lower levels and had higher thresholds and less influence on the yogurt flavor (Tian et al., 2020). Pyrazines and pyrroles at low concentrations have specific aromas and can contribute prominently to the overall flavor of fermented milk. Among these compounds, pyrazines mainly produce a barbecue-like flavor. Among all pyrazine compounds, 2,6-dimethylpyrazine, 2-methylpyrazine, and 2,5-dimethylpyrazine were detected as flavoring agents in all samples. The concentration of 2-methylpyrazine was cumulative throughout the fermentation process. The concentration of volatile compounds in both 2,5-dimethylpyrazine and theophylline varied irregularly, with the lowest concentration detected after 48 h of fermentation. These heterocyclic compounds contributed to the caramelized and pasty flavors, as well as the overall flavor of fermented milk.

3.2.2. Sensory evaluation

The sensory quality of fermented milk was evaluated at each time point throughout the fermentation process. Seven sensory attributes obtained from the pre-selection were scored, and the results are presented as radar charts (Fig. 3A). The sensory evaluation scores of the samples fermented for 3–12 h were close to one another, and the viscosity characteristics scores were low, which is in line with the fermentation pattern. The initial state of the samples was mainly fluid and did not drastically change due to the absence of complexes. The bacteria in the control samples had not yet started to act and the 0-h fermentation samples could be considered as reconstituted milk powder and therefore qualitatively different from fermented milk in terms of organoleptic properties. After more than 24 h, the state of fermented milk was mainly solid. The degree of sedimentation of fermented milk was closely related to the fermentation time. Depending on the solid-liquid equilibrium point, less sedimentation was observed at the beginning of the fermentation, and the sedimentation increased with time. The accumulation of fermentation aroma increased with time and reached its peak after 48 h. The color of fermented milk received the highest rating after 12 h and then stabilized after 24 h.

Correlations between volatile compounds and sensory attributes during fermentation were analyzed. Fig. 4B shows the Pearson correlation coefficients between volatiles and odors. The correlation between volatile compounds and odor sensory attributes is shown in the upper right corner, and the significance level is indicated by an asterisk. The interacting lines at the lower left corner indicate the correlation between volatile compounds and odor sensory attributes: the thicker the line, the larger the R-value and the stronger the correlation (Wang et al., 2024). Correlations ($P < 0.01$) between fermented aroma and 2-nonanal (cucumber, fat, green), 2-undecanone (orange, fresh, green), 2-pentadecanone, acetic acid (sour), hexanoic acid (sweat), octanoic acid (sweat, cheese), and lauric acid (metal). Correlations ($P < 0.05$) were observed between fermented aroma and 3-methyl-2-butenal, trans-2-octenal (green, nut, fat), 2-tridecanone, isobutyl butyrate, ethyl caprylate (fruit, fat), decanoic acid (rancid, fat), n-hexanol (resin, flower, green), 1-octen-3-ol (mushroom), 2-nonanol (cucumber), Linalool (flower, lavender), geraniol (rose, geranium), dodecanol (fat, wax), 2-methylpyrazine (popcorn), and butylhydroxytoluene (Fang et al., 2023; Wang et al., 2024).

Only one compound, 1-octen-3-ol (mushroom), was found to be significantly positively correlated ($P < 0.05$) with the fermentation off-flavors. 1-Octen-3-ol, a secondary alcohol with a mushroom and earthy odor formed through the hydrogen peroxide decomposition of 10-hydroperoxide isomer of linoleic acid, provided an unpleasant odor to fermented milk (Wurzenberger and Grosch, 1984).

3.3. Fermentation characteristics of fermented milk with prebiotics

As shown in Fig. 4E, the pH trends of fermented milk with/without prebiotics were consistent. The differences between the control group and the inulin group were small, and the pH values in the final stabilization stage were slightly higher than those in GOS and Inulin/GOS group. We speculated that the addition of GOS might have a greater effect on the acidification process. A reduction in pH triggers protein denaturation, unfolding of protein-peptide chains, and charge neutralization, ultimately leading to the formation of gel structures (Nsogning

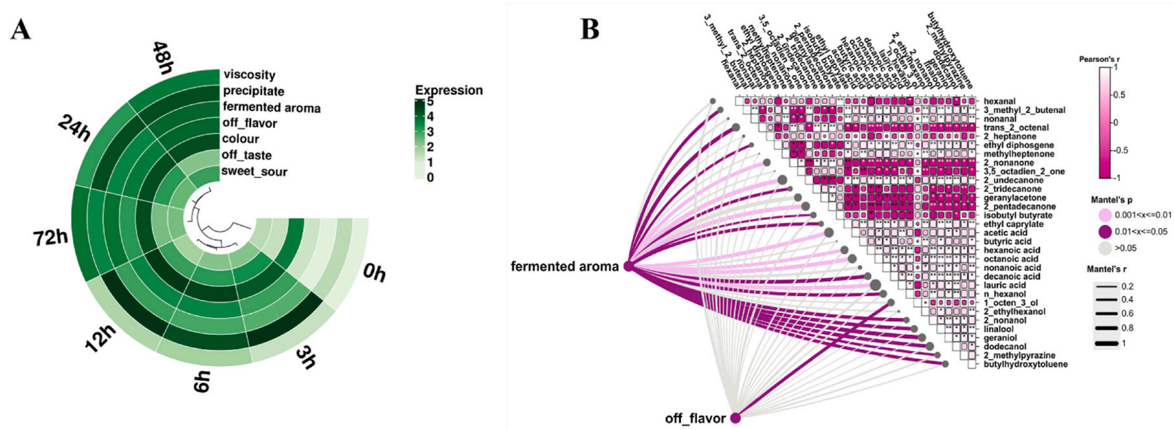

Fig. 3. Heatmap based the results of sensory evaluation of fermented milk during fermentation **(A)**; Correlation between sensory properties and volatile compounds **(B)**.

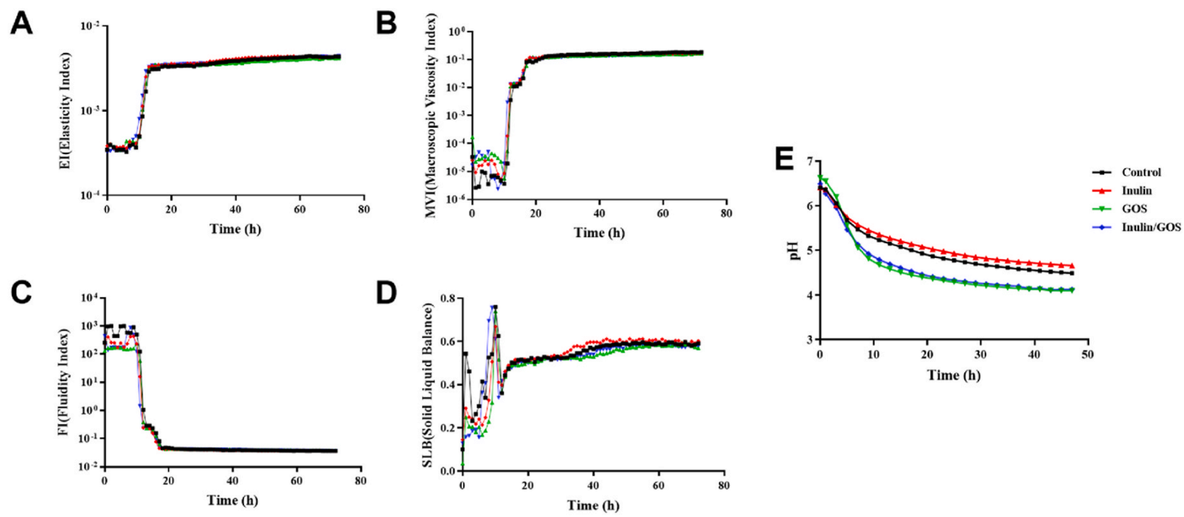

Fig. 4. Microrheological properties and pH of fermented milk added prebiotics during fermentation. Elasticity index **(A)**; Macroscopic Viscosity Index **(B)**; Fluidity Index **(C)**; Solid-Liquid Balance **(D)**; pH **(E)**. Legend: Black: Control; Red: Inulin; Class A; Green: GOS; Blue: Inulin/GOS. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

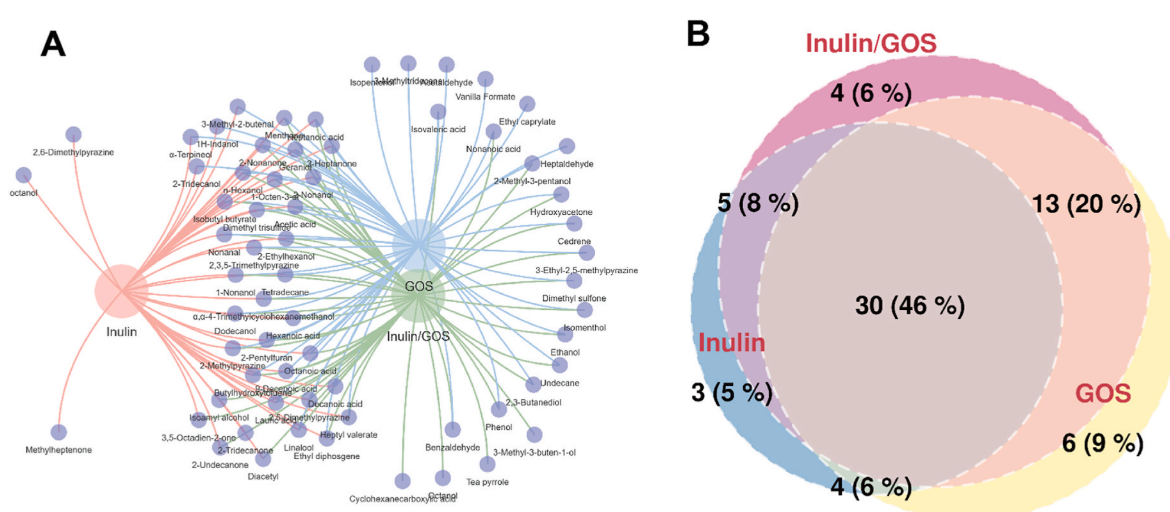

Fig. 5. Characterizing the composition of volatiles in three probiotic fermented milks. Correlation network diagram **(A)**; Venn diagram **(B)**.

Dongmo et al., 2016).

The effects of the addition of prebiotics on the rheological properties of fermented milk are shown in Fig. 4A–D. The elasticity, viscosity and fluidity indexes of fermented milk before and after the addition of prebiotics followed the same trend, and the values were close to one another without significant changes. The SLB point of fermented milk in the inulin group was the first to reach 0.5, and the SLB value of the inulin group was slightly higher than that of other groups. In conclusion, the addition of inulin might have advanced the solidification time, and the elasticity of the fermented milk was slightly higher than that of other groups.

3.4. The performance of flavor in fermented milk with prebiotics

3.4.1. GC-MS

As can be seen from the volatile compound network visualization and Venn diagrams (Fig. 5), the composition of compounds in fermented milk with the addition of different prebiotics did not dramatically change and was nearly half the compounds commonly detected in all three groups. Most of the intersections between the GOS and inulin groups were alcohols, most of the compounds shared by both the inulin/GOS and inulin groups were ketones, and alcohols shared by both the GOS and inulin/GOS groups were most prevalent.

The addition of prebiotics can change the composition of VOCs, as well as the content of some key flavor substances. To investigate the effect of prebiotics on the flavor release of fermented milk, control group (no prebiotics added), inulin-added, GOS-added, and inulin/GOS-added samples were sampled after 48 h of fermentation, and the concentrations of volatiles in the samples were identified by HS-SPME/GC-MS (Table S2). Diacetyl, acetaldehyde, and acetoin are the typical odor-active substances in yogurt, and when presented at the right concentrations, these key compounds generate an attractive flavor (Tian et al., 2020). With the addition of prebiotics, especially to fermented milk with inulin, the concentration of acetic acid and butyric acid is significantly reduced, and the irritating acidity is weakened, which leads to the softer overall flavor of fermented milk. Fermented milk with the addition of inulin had substantially higher concentrations of 2-ethylhexanol (rose, green spice), linalool (floral, lavender), 2,5-dimethylpyrazine (cocoa, roasted spices), and 2,3,5-trimethylpyrazine (roasted spices, potatoes) compounds, especially the first two, which are often thought to be associated with favorable. The greatest increase in the content of 2-nonanol and linalool, which are compounds strongly correlated with the fermentation aroma, was observed in fermented milk with the addition of inulin. At the same time, a significant decrease in 2-pentylfuran, which has been described as a stimulating, bean and butter flavor, was observed. The addition of prebiotics significantly reduced the content of 1-octen-3-ol, which is strongly associated with off-flavors. Disclosing the phenylalanine in fermented milk undergoes Strecker degradation to form large amounts of benzaldehyde during the pre-fermentation phase. As the pH values decreased during fermentation progress, lactic acid bacteria continue to proliferate.

3.4.2. Sensory

To fully evaluate the quality of fermented milk with the addition of probiotics, sensory of volatile compounds is another important reference index in addition to the composition and characteristics. Fig. 6A demonstrates the results of sensory evaluation after the addition of prebiotics. Prebiotics affected the sensory scores by improving the taste, aroma and odor, and tissue state. Viscosity, as the main textural characteristic of fermented milk, was significantly changed by the addition of inulin prebiotics (Fig. 6A). Several studies reported that inulin was regarded as promising candidates for yoghurt thickeners, because they were able to stabilize dairy products by the formation of soluble protein-polysaccharide complexes and the increase of viscosity (Bedani et al., 2013; Crispín-Isidro et al., 2015). Inulin enhances the structure of the hydrogel network, whereas the addition of galactose may interfere with the gel network. This is consistent with the analysis of a previous study (Galli et al., 2022), where the gel structure of yoghurt with added GOS showed more pores, but smaller pores, indicating a weaker gel. At present, studies on the utilization of GOS by LAB mainly focus on their use as all or part of a carbon source to analyze their growth-promoting effects (Lamsal, 2012; López-Sanz et al., 2018). Multiple biological functions of GOS have been demonstrated in infants and adults (G.T. Macfarlane et al., 2008). However, the performance of GOS seems to be unsatisfactory in terms of sensory quality improvement. This is possibly cause of the inulin group having the highest overall rating and being favored by most evaluating experts.

Compared to the control group, the sweet-sour ratio, taste off-flavor and color of fermented milk with the addition of prebiotics were improved to different degrees. The only group with higher elasticity and sedimentation scores than the control group was the inulin group, which could be attributed to the reduction of sedimentation and the better overall texture of fermented milk, as well as the improved elasticity. After 48 h of fermentation, off-flavors were minimal in the inulin and inulin/GOS groups, and the fermentation aroma was richer in the inulin group.

The total sensory evaluation scores were improved after the addition of prebiotics to the fermentation, with the inulin group having the

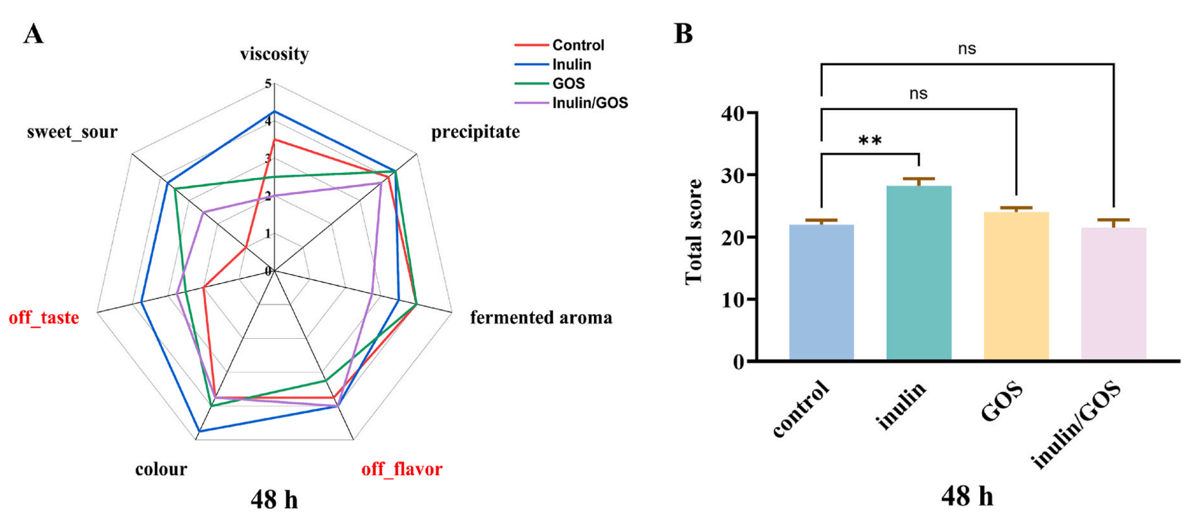

Fig. 6. Sensory evaluation results of fermented milk with prebiotics **(A)**, Rada chart of attributes at fermented 48 h **(B)**. Significant difference between two groups: "ns" indicates no significant difference, $^{**}P < 0.01$.

highest total score. Additionally, the overall sensory quality of fermented milk with the addition of inulin was significantly higher than that of the control group ($P < 0.01$) (Fig. 6B). According to the results on volatile compounds, the addition of prebiotics may promote the release of flavor substances in the matrix of fermented milk samples and to enrich their flavors, especially inulin. These results suggest that the potential advantages associated to the use of inulin prebiotics would not be limited to the improvement of growth and fermentation kinetics as previously proposed but include also the textural characteristics of fermented milks. They also open the possibility of using similar prebiotics to improve the sensory quality of fermented dairy products.

4. Conclusions

This study aimed to track the variations of the volatile components and rheological properties in *Streptococcus thermophilus* fermented milk throughout the fermentation process and explore prebiotic fermented milk from a sensory perspective. Fermented milk produced using *Streptococcus thermophilus* as a fermenting agent had stable quality after 40 h of acidification, and the highest fermentation aroma and the weakest off-flavor were reached at 48 h of fermentation. Benzaldehyde, 2-undecanone, caprylic acid, n-hexanol, and 2-nonanol were the main active flavors during fermentation. Compared with GOS and inulin/GOS, inulin prebiotics significantly improved the texture and flavor release of fermented milk. To some extent, these results provide new insight into the dynamic changes of fermented milk during fermentation, future research should explore a wider range of LAB in fermented milk and delve into multiple prebiotics to deepen our understanding of fermented milk production.

CRediT authorship contribution statement

Xuelu Chi: Conceptualization, Methodology, Investigation, Writing – original draft. **Qingyu Yang:** Writing – review & editing. **Yufang Su:** Conceptualization, Writing – review & editing. **Yanmei Xi:** Writing – review & editing. **Weizhe Wang:** Writing – review & editing. **Baoguo Sun:** Conceptualization, Writing – review & editing. **Nasi Ai:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100839.

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