



The influence of soy protein hydrolysate (SPH) addition to infant formula powder on *Streptococcus thermophilus* proliferation and metabolism

Zhi-hui Sun^a, Ming-jing Yao^{a,c}, Xin Bian^a, Qing-qi Guo^b, Hua-nan Guan^a, Yang Yang^a, Bing Wang^a, Yan-guo Shi^a, Wojciech Piekoszewski^{e,f}, Xiao-wan Yang^{a,d}, Na Zhang^{a,*}

^a Key Laboratory of Food Science and Engineering of Heilongjiang Province, Harbin University of Commerce, 36# No.138, TongDa Street, DaoLi District, Harbin 150076, PR China

^b Forestry School, Northeast Forestry University, No.26, HeXing Street, XiangFang District, Harbin 150040, PR China

^c Shandong Provincial Key Laboratory of Food and Fermentation Engineering, Shandong Food Ferment Industry Research & Design Institute, Qilu University of Technology (Shandong Academy of Sciences), No.41, JieFang Road, LiXia District, Jinan 250013, PR China

^d Shanghai Special Favor Biotechnology Co., Ltd, No. 56, JunGong Road, Yangpu District, Shanghai 200433, PR China

^e Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, 2 Gronostajowa, 30-868 Krakow, Poland

^f School of Biomedicine, Far Eastern Federal University, FEPU Campus, Russian Island, Vladivostok, Russian Federation

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ABSTRACT

Nowadays, more and more infants are getting allergic to cow's milk protein, so it is urgent to search for infant formula powder with milk protein alternatives. In the present work, soy protein hydrolysate (SPH) was added to protein-free infant formula powder and the effects of SPH addition on proliferation and metabolism of *Streptococcus thermophilus* were studied. Compared with commercially available infant formula powder (CK) and protein-free milk powder (BK), the infant formula powder with 20% SPH significantly enhanced the proliferation of *S. thermophilus* in MRS medium, resulting in a higher cell density and greater viable counts. Moreover, the influence of SPH on the metabolism of *S. thermophilus* was investigated by analyzing the content of seven organic acids and H₂O₂ in the medium. The higher content of organic acids and H₂O₂ is consistent with the stronger antibacterial activity to *Escherichia coli*. As a consequence, the addition of SPH to infant formula powder can effectively promote the growth of probiotics and SPH may be a promising protein alternative in the infant formula powder.

1. Introduction

For newborn infants, breast milk can provide all essential nutrients in their first months and is considered to be the golden standard for nutrition (Walker, 2010). However, for a number of legitimate reasons not all newborns can be breastfed and have to be either totally or partly fed with infant formulas derived from cow milk (Akkerman, Faas, & de Vos, 2018). Unfortunately, due to the immature gastrointestinal and immune systems, 5%–15% of infants are intolerant of cow's milk protein especially casein and 2%–7.5% of them occurs a true allergy (Green Corkins & Shurley, 2016). The best solution for the problem is to find an alternative formula for cow's milk.

So far, lots of efforts have been made to find the alternative for cow's

milk, such as soy formula and partially hydrolyzed cow's milk protein formula. At first, each of them is a good choice. However, with time, it is found soy formula exerts little influence on managing symptoms of cow's milk protein allergy and the American Academy of Pediatrics recommends against its use for the infants who are allergic to cow's milk (Bhatia, Greer, & American Academy of Pediatrics Committee on, 2008). Partially hydrolyzed cow's milk protein formula, which is obtained by partially hydrolyzing casein with whey or hydrolyzing 100% whey protein, can effectively alleviate feeding intolerance but has no effect on preventing atopic disease (Green Corkins & Shurley, 2016; Boyle et al., 2016; Greer, Sicherer, Burks, Comm, & Section, 2008). In light of Food and Drug Administration (FDA) standards, extensively hydrolyzed protein infant formulas whose molecular is much lower, are considered

Abbreviations: SPH, Soy protein hydrolysates; CK, Control group; BK, Blank group; FDA, Food and Drug Administration; SPI, Soy protein isolate; WPH, Whey protein hydrolysates; CH, Casein hydrolysates.

* Corresponding author at: Key Laboratory of Food Science and Engineering, Harbin University of Commerce, No. 36, College of Food Engineering, Harbin University of Commerce, No. 138, Tongda Street, Daoli district, Harbin, Heilongjiang Province 150076, PR China.

E-mail address: foodzhangna@163.com (N. Zhang).

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hypoallergenic, less likely to cause an anaphylactic reaction and effective in preventing allergies (Green Corkins & Shurley, 2016). However, compared with standard formulas, extensively hydrolyzed cow's milk protein formulas are much more expensive and less toothsome (Joeckel & Phillips, 2009). It is urgent to find an economical, tasty, allergen free infant formula.

Soybean protein hydrolysate (SPH) is much cheaper and tastier and maybe a promising substitute for milk in infant formula. SPH is mainly composed of low molecular peptides which are easily digested and absorbed. Moreover, it has been discovered that peptides can stimulate the proliferation of probiotics (Yu, Amorim, Marques, Calhau, & Pintado, 2016). Probiotics is a microbial supplement that is helpful to host health by improving intestinal microbial balance (Afric, 1989; Delgado-Fernández, Hernández-Hernández, Olano, Moreno, & Corzo, 2020), and plays an important role in infant intestinal flora (Helena, McCartney, & Glenn, 2007). It can be used as an effective tool to alleviate food allergy (Lourens-Hattingh & Viljoen, 2001). Probiotic can decompose hexose to produce lactic acid, improving lactose intolerance and regulating the pH of the digestive system (Leis, de Castro, de Lamas, Picans, & Couce, 2020; Talwalkar & Kailasapathy, 2004), and antagonize pathogens by producing antimicrobial substances or competitive exclusion, effectively inhibiting the adhesion and growth of pathogenic bacteria in the intestine (Kailasapathy & Chin, 2000; Parvez, Malik, Ah Kang, & Kim, 2006). However, probiotics cannot synthesize some amino acids by themselves, such as leucine, valine, and histidine, which limits its rapid proliferation (Sah, Vasiljevic, McKechnie, & Donkor, 2014).

Adding peptides to the medium could relieve the nutrition limitation. For example, soybean peptides, obtained by protease, papain or flavor protease, can effectively stimulate the proliferation of probiotics, including *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, regulating the intestinal microecological of humans and animals (Sah et al., 2014). Besides promoting the proliferation of probiotics, protein hydrolysates can also be metabolized and inhibit the growth of harmful bacteria (Chen, Jheng, Shyu, & Mao, 2013; Pridmore, Pittet, Praplan, & Cavadini, 2008). Some protein hydrolysates could promote the growth of probiotic to inhibit the growth of some pathogens, such as *Staphylococcus aureus* and *Escherichia coli*. It is confirmed that the inhibition effect of lactic acid bacteria is due to the bacteriostatic substances, including organic acids (lactic acid and acetic acid), hydrogen peroxide and antimicrobial peptide (lactobacillin, enterococin, and so on) which have the effect of inhibiting related species.

In this paper, soybean protein hydrolysates (SPH) were added into protein-free infant milk powder. The object of this research was to assess the influence of the SPH infant formula on the proliferation and metabolism of *Streptococcus thermophilus* KLDS3.020, one of the most common probiotics, and the inhibition of *Escherichia coli* BL21.

2. Materials and methods

2.1. Materials

Soy protein isolate (SPI) was supplied by Shandong Yuwang Industry Co., Ltd. (Shandong, China). Alcalase 2.4 L (P4860) (208, 000 U/g) was obtained from Sigma, USA. *Streptococcus thermophilus* KLDS3.020, and *Escherichia coli* BL21 were obtained from Key Laboratory of Food Science and Engineering Harbin University of Commerce. Commercial milk powder (Nestle Foods Co Ltd) was purchased from Carrefour supermarket. There are 18 g protein, 11 g soybean oil, 10 g corn oil, 8 g palm oil, 24 g lactose, 1.5 g galacto-oligosaccharide, 26 g maltodextrin and other 1.5 g microelement in the milk powder. Analytical standard-grade formic, oxalic, malic, acetic, tartaric, lactic, citric acid were purchased from sigma, USA. The other chemical reagents were of analytical grade.

2.2. Preparation of SPH and SPH-infant formula powder

An SPI solution was made up to a concentration of 5% w/v protein by

dissolving SPI in deionized water. The temperature was set 55 °C for the hydrolysis and the pH was adjusted to pH 8.0 by addition of 2 M of NaOH. Alcalase® was added at a ratio of 1500 U/g in SPI solution. After 3 h, the reaction was quenched by heating at boiling water. Afterwards the pH was adjusted to 4.6. The hydrolysates were then centrifuged at 8,000 r/min for 15 min. The supernatants obtained were spray dried (DZF-6020, Shanghai Yiheng Technology Co., LTD) and kept in a desiccator for further use. According to Kjeldahl method, the trichloroacetic acid-soluble nitrogen index (TCA-NSI) of the prepared SPH was 92.16%±0.67% (GB 5009.5–2016). The degree of hydrolysis (HD) of the prepared SPH was 29.13%±1.37%, detected by the method of Alder-Nissen (Adlernissen, 1986). The molecular weight of the prepared SPH mainly distributed below 14.4 kDa, assayed by the method of SDS-PAGE.

Infant milk powder (protein-free), all the raw materials of which were supplied by Nestle Foods Co Ltd, was based on the "National Food Safety Standard for Infant Formula Food.

SPH-infant formula powder was prepared by adding 5%, 10%, 15%, 20%, 25% (w/v) SPH to infant milk powder (protein-free).

2.3. Determination of growth of probiotic bacteria

The infant powders without SPH were used as the blank group (BK), while those with commercial infant formula were used as the control group (CK). Infant powder with 5%, 10%, 15%, 20% and 25% (w: w) SPH were used as the experimental group. Then 2% infant powder (w: v) was added to MRS medium. The mixture was sterilized at 121 °C for 15 min, inoculated and incubated at 37 °C for 24 h.

The growth of probiotic bacteria was determined by the turbidimetry method (Trinder et al., 2016). During the incubating period, the optical density (OD) value of culture medium of 0 h, 6 h, 12 h, 18 h, 24 h were measured.

The live bacteria count was determined by the plate-counting method (Batista et al., 2017). 0.1 mL bacterial fluid was dipped on the 0 ~ 25% SPH medium and commercial milk powder medium, respectively. Then incubate at 37 °C for 24 h.

2.4. Evaluation the inhibitory effect of *Streptococcus thermophilus* KLDS3.020 on *E. coli* BL21

Oxford cup method was used to test the inhibitory ratio of *Streptococcus thermophilus* KLDS3.020 culture on *E. coli*. The probiotic was cultured in MRS medium supplemented with infant formula which included SPH ranged from 5 to 25% for 24 h. (Somsap, Bangrak, Bhoopong, & Lertcanawanichakul, 2016).

2.5. Determination of titration acidity

Titration acidity was measured according to the method of Hofvendahl & Hahn-Hägerdal (Hofvendahl & Hahn-Hägerdal, 2000). The titration acidity of the culture fluid was measured at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h during growth of the *Streptococcus thermophilus* culture.

2.6. Determination of organic acid

Compared with the basic medium (MRS medium), 15% SPI and 15% SPH were added respectively to the basic medium. The 15% SPI group, 15% SPH group and the basic medium were used as control group, sample group and blank group respectively. The content and composition of organic acids in liquid media were determined by HPLC. Samples were adjusted to pH 3.5 by 20% H₂SO₄, subjected to centrifugation at 13,000 × g for 4 min, then the supernatant was filtrated through 0.45 μm cellulose acetate filters (Sigma, USA).

An HPLC system (Waters® 2695, USA), equipped with a manual sampler, a photodiode array detector (Waters® 2489), and Empower®

Table 1
Standard curves used for organic acid quantification.

Peak number	Organic acid	Retention time (min)	Equation of linear regression	R ²	Range (mg/L)
1	Formic acid	2.403	y = 80640x + 3.210	0.981	20–200
2	Oxalic acid	2.745	y = 3543.7x + 14.869	0.988	10–100
3	Malic acid	2.957	y = 2199.22x + 0.9188	0.992	50–500
4	Acetic acid	3.594	y = 139.02x + 0.7112	0.996	100–1000
5	Tartaric acid	3.957	y = 467.63x - 1.565	0.973	20–200
6	Lactic acid	4.567	y = 1750.12x + 0.8043	0.999	80–800
7	Citric acid	6.309	y = 1884.03x - 0.1525	0.987	30–300

Chromatography Workstation software, was used for the identification and quantification of organic acids. HPLC separation was performed with isocratic elution (0.1% v/v H₃PO₄ and methanol) on an Agilent ZORBAX SB-C18 column (250 mm × 4.6 mm I.D., 5 μm particle size) at a flow rate of 1 mL/min. Absorbance was monitored at 210 nm and the sample injection volume was 10 μL (Yuchen et al., 2019). Identification and quantification of organic acid were carried out according to the retention time and the external standard solution calibrations, and the standard curves of seven organic acids were shown in Table 1.

2.7. Determination of hydrogen peroxide content

Titanium salt colorimetry method was used to determine the content of H₂O₂ (X. Liu et al., 2011). Hydrogen peroxide and titanium ions can form a stable orange complex in acidic solution, which has an absorbance at 430 nm.

2.8. Statistical analysis

Data from all the experiments in this paper were statistically analyzed by one-way ANOVA with one variable being procedure treatment. All assays were carried out in triplicate. Results were reported as mean ± standard deviation (SD).

One-way ANOVA and correlation analysis were performed using the SPSS 13.0 software (version 20, SPSS Inc., Chicago, USA). Differences were considered significant if $P < 0.05$ in the Tukey test.

3. Results and discussion

3.1. Effect of SPH on the growth of *Streptococcus thermophilus*

The optical density (OD₆₀₀) of *Streptococcus thermophilus* during 24 h, incubated in BK group, CK group, and experimental group, are shown in Fig. 1 A. The OD₆₀₀ of SPH group was higher than that of BK group all the time. Compared with CK group, both the 20% and 25% SPH group are very effective on the proliferation of *S. thermophilus*. In 20% SPH group, the OD₆₀₀ reached the highest at 24 h, which was 1.20 and 1.05 times of BK and CK group, respectively.

Infant formula with different SPH concentrations were supplemented to the media, for the purpose of evaluating the influence of SPH on the viable cells count of *S. thermophilus*. After culturing for 24 h, when the concentration of SPH was 0%, the viable count of *Streptococcus thermophilus* was 10.3×10^6 (CFU/mL). When the concentration of SPH increased to 0%, the viable count increased to 18.9×10^6 (CFU/mL) ($P < 0.05$). However, when SPH concentration exceeded 20%, it did not promote the growth of *S. thermophilus* any more ($P > 0.05$). Also, there were no significant differences between 15% and 20%. However, viable

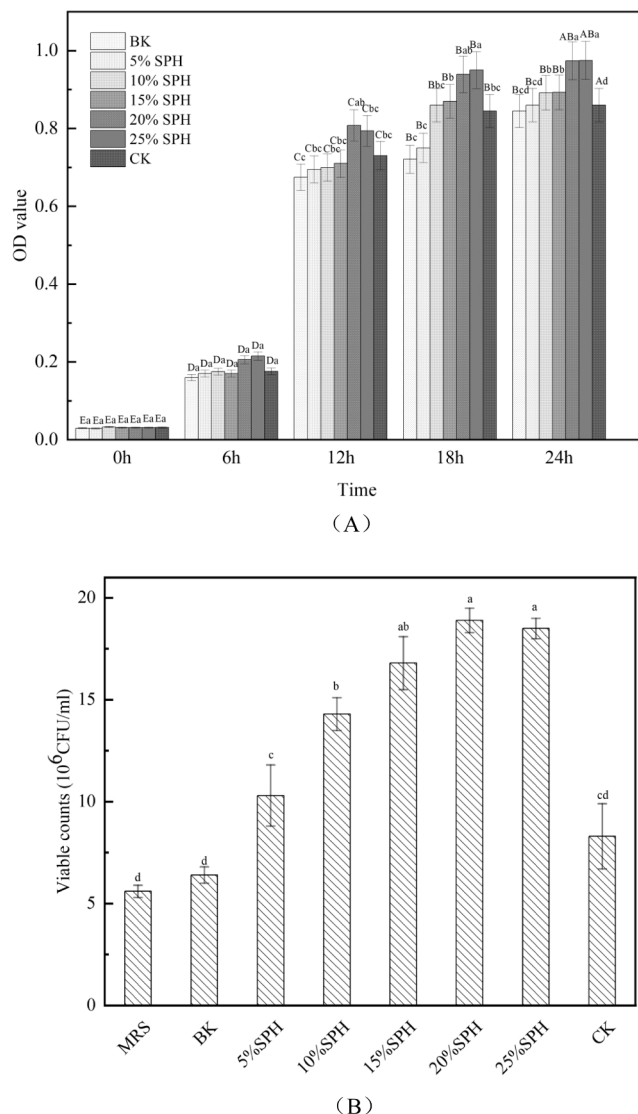


Fig. 1. The effect of SPH addition to infant milk powder on the growth of *Streptococcus thermophilus* (A) Changes of OD values during 24 h (B) The viable count of *S. thermophilus* at 24 h. In the figure, SPH refers to soy protein hydrolysate, BK refers to the protein-free milk powder without SPH, CK refers to commercially available infant formula milk powder, MRS refers to MRS alone without infant milk powder and X% (X = 5,10,15,20,25) SPH refers to X% SPH addition to infant milk powder. In the figure, different uppercase letters and lowercase letters indicate significant differences in OD value and viable counts among the whole growth and within different culture medium, respectively, according to Tukey's test ($P < 0.05$).

cells in SPH media were significantly greater than those of the BK and MRS alone ($P < 0.05$) (Fig. 1 B). Furthermore, viable cells in media containing SPH of at any level were significantly greater than those of the MRS alone, the BK and CK group ($P < 0.05$).

Lactic acid bacteria have numerous nutritional requirements for growth, especially nitrogen sources (Guarner, 1998; Law, Sezgin, & Sharpe, 1976). It cannot assimilate inorganic nitrogen sources, therefore, they must hydrolyze proteins and peptides to meet cell anabolism for amino acids. Various substances are proved to enhance the growth of certain lactobacillus (Ravula & Shah, 1998), such as whey protein hydrolysates (WPHs), which contain more easily attainable nitrogen sources. Thus, WPHs can stimulate the proliferation of lactobacillus (Mccomas & Gilliland, 2003). So do casein hydrolysates (CH) and whey protein hydrolysates (WPHs). Also, when they were added into fermented milk, total probiotic cell counts of mixed strains of

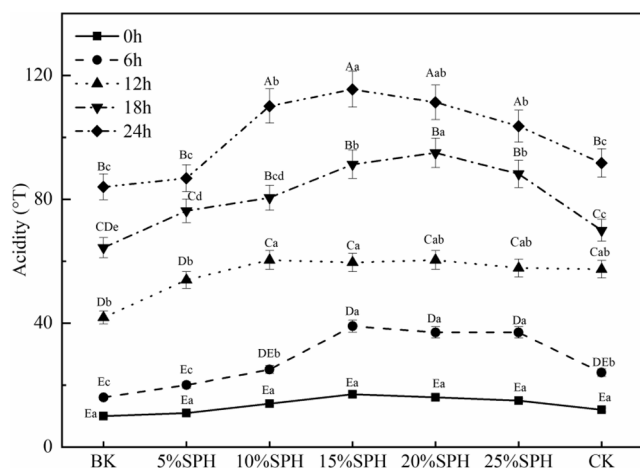


Fig. 2. The effect of soy protein hydrolysate (SPH) addition to milk powder on the titratable acidity during 24 h. In the figure, SPH refers to soy protein hydrolysate, BK refers to the protein-free milk powder without SPH, CK refers to commercially available infant formula milk powder, and X% (X = 5,10,15,20,25) SPH refers to X% SPH addition to infant milk powder. In the figure, different uppercase letters and lowercase letters indicate significant differences in acidity among the whole growth and within different culture medium, respectively, according to Tukey's test ($P < 0.05$).

S. thermophilus ST-7 and *L. acidophilus* LA-5 increased (Lucas, Sodini, Monnet, Jolivet, & Corrieu, 2004; Dave & Shah, 1998). Nevertheless, WPH exerts no significant effect on the proliferation of *L. bulgaricus* 18 (Mccomas & Gilliland, 2003). This indicates that the effect of protein hydrolysate on lactic acid bacteria depends on the species of bacteria and the protein sources. In this work, we studied the influence of soy protein hydrolysates (SPHs) on the proliferation of *S. thermophilus*. From the above, we can know that SPHs can significantly promote the proliferation of *S. thermophilus* ($P < 0.05$).

3.2. Inhibitory of *Streptococcus thermophilus* to *E. coli*

Bacteriostasis and acid-producing ability are important characters of *S. thermophilus* and they are closely connected with the growth of *S. thermophilus*. Therefore, the effect of SPH on the bacteriostasis and acid-producing ability of this probiotic were evaluated. *S. thermophilus* was incubated in CK group, BK group and SPH group for 24 h, and the change of titratable acidity were shown in Fig. 2. Compared with CK group, SPH groups showed higher titratable acidity. This was consistent with the result of Fig. 1, SPH could remarkably ($P < 0.05$) increase the growth of *S. thermophilus*. At the end of the culture (24 h), the titratable acidity of 15% SPH group reached 115.5 ± 3.41 °T, which was 1.37 times and 1.26 times of the BK group (84.0 ± 4.23 °T) and CK group (91.7 ± 4.58 °T), respectively. The results illustrated that the supplementation of SPH could decrease pH (data not shown) and increase titratable acidity. Therefore, SPH was a good choice as a nitrogen ingredient for *S. thermophilus*.

Table 2 summarizes the results of the test for antimicrobial activity.

Table 2
Inhibitory effect of various concentrations of SPH (5–25%) against *E. coli*.

Group	BK	5%	10%	15%	20%	25%	CK
Graph							
Inhibitory Ring (mm)	8.53 ± 0.2^c	9.96 ± 0.3^c	11.33 ± 1.4^b	15.52 ± 0.5^a	15.46 ± 1.1^a	15.33 ± 0.5^a	9.96 ± 0.4^c

In the table, BK refers to the protein-free milk powder without SPH, CK refers to commercially available infant formula milk powder, and X%(X = 5,10,15,20,25) SPH refers to X% SPH addition to infant milk powder. Different lowercase letters in the line indicate significant differences between bland group, control group and experimental group according to Tukey's test ($P < 0.05$).

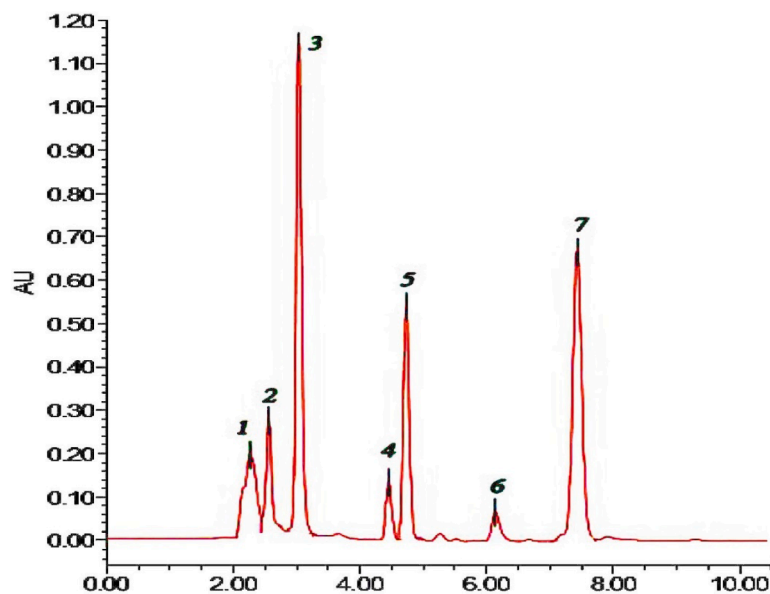
Compared with BK and CK groups, the addition of SPH, no matter what dosage it was, could significantly enhance the antimicrobial activity ($P < 0.05$). Among all the experimental groups, the 20% SPH group showed the highest antimicrobial activity. The results suggest that SPH addition could enhance antimicrobial activity in vitro.

It has been reported that the existence of an enzyme system in lactobacillus that can decompose protein hydrolysates is necessary for its proliferation (Liu J., et al., 2017; Van Niel & Hahn-Hägerdal, 1999). There are three main components in the enzyme system: enzymes that degrade casein to form polypeptide fragments, transporters that transport oligopeptides into cells, and enzymes that degrade polypeptides into amino acids within cells (Van Niel & Hahn-Hägerdal, 1999). Among them, the transporter is consisted of an oligopeptide transport system, which helps oligopeptides to transport across the cell membrane. As to *S. thermophilus*, oligopeptides is more suitable than free amino acids to satisfy its all amino acid requirements. Therefore, the addition of SPH can promote the growth and acid activity of this probiotic.

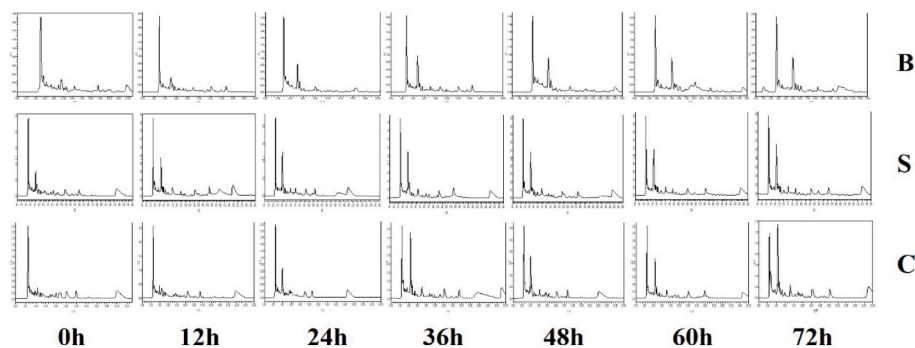
3.3. Effect of SPH on the metabolism of *Streptococcus thermophilus*

It has been reported that protein hydrolysate can affect the metabolism of lactic acid bacteria, especially the organic acids and H_2O_2 which can strengthen the antibacterial ability. Therefore, the content of seven common organic acids (Fig. 3) and H_2O_2 were tested to study the influence of SPH on the metabolism of *S. thermophilus*. Tables 1 and 3 shows the standard linear curve of seven organic acids and the changes of organic acid content during 72 h, respectively. In Table 3, seven organic acids increased significantly from 0 h to 72 h in all groups ($P < 0.05$). It appears that the acetic and lactic acids were produced by the *S. thermophilus* during the fermentation. Formic acid, citric acid and oxalic acid were produced in a small amount. In a word, the content of acetic acid in sample group increased significantly during the incubation period. Compared with the blank and control group, significant increase ($P < 0.05$) of acetic acid content was observed during the first 48 h. However, there was no significant difference ($P > 0.05$) on acetic acid content between sample group and blank group in the last 24 h of incubation. As is shown in Table 3, there was a steadily increase of lactic acid content in sample group during the incubation period. What's more, in sample group, lactic acid was generated significantly less ($P < 0.05$) during the first 12 h than the blank group. At 72 h, the sample group showed the highest lactic acid content. The present results suggest that the SPH media could promote bacteria metabolism which leads to organic acids growth (Fig. 2). The H_2O_2 content in SPH group is 109.30 ± 2.34 mg/mL, which was 6.94 times as much as the SPI group (15.73 ± 0.61 mg/mL) and 11.23 times as much as the MRS media (9.73 ± 0.25 mg/mL).

This is because of the strong hydrolysis ability of alkaline protease, which can produce more low molecular peptides. The peptides facilitate the proliferation of *S. thermophilus* and promote the bacteria to produce organic acids (Goto et al., 2015). The results of our study are in line with previous reports. The lactic acid content was significantly increased with the supplement of soy protein hydrolysate and in the sample group the content of lactic acid was 1.8 times of the blank group when cultured to 60 h (Mark Hsieh, Yang, & Iannotti, 1999). The lactic acid productivity



(A)



(B)

Table 3
Changes of the content of seven organic acids during the growth of *S. thermophilus*.

Organic acid	Group	Time						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Oxalic acid	S	73.91 ± 3.14 ^{A,f}	97.12 ± 4.21 ^{B,e}	116.18 ± 4.03 ^{B,d}	130.68 ± 2.19 ^{B,c}	140.59 ± 4.15 ^{B,c}	161.09 ± 4.27 ^{B,b}	231.52 ± 9.23 ^{A,a}
	C	65.56 ± 2.35 ^{A,f}	107.08 ± 6.40 ^{A,e}	136.56 ± 8.12 ^{A,d}	148.82 ± 3.09 ^{A,d}	179.18 ± 3.02 ^{A,c}	216.76 ± 3.12 ^{A,b}	237.10 ± 8.10 ^{A,a}
	B	66.20 ± 4.56 ^{A,f}	96.87 ± 5.67 ^{B,e}	109.71 ± 7.89 ^{C,d}	114.03 ± 5.67 ^{C,d}	130.10 ± 8.90 ^{C,c}	154.03 ± 4.76 ^{B,b}	156.10 ± 5.34 ^{B,a}
Malic acid	S	364.89 ± 5.18 ^{B,e}	840.56 ± 6.78 ^{A,d}	848.12 ± 8.24 ^{B,c}	1222.03 ± 12.31 ^{A,c}	1797.93 ± 4.56 ^{A,b}	1823.82 ± 5.67 ^{A,b}	2389.97 ± 3.42 ^{A,a}
	C	414.13 ± 3.64 ^{A,g}	636.30 ± 7.90 ^{B,f}	810.05 ± 2.45 ^{B,e}	1142.21 ± 8.41 ^{B,d}	1169.30 ± 2.34 ^{B,c}	1419.26 ± 6.78 ^{B,b}	1669.73 ± 7.21 ^{B,a}
	B	286.02 ± 8.95 ^{C,f}	875.05 ± 10.23 ^{A,e}	1107.02 ± 3.34 ^{A,d}	1121.89 ± 3.68 ^{B,d}	1203.91 ± 9.21 ^{B,c}	1211.81 ± 8.91 ^{C,b}	2084.60 ± 7.89 ^{A,a}
Acetic acid	S	175.09 ± 6.71 ^{B,f}	491.065 ± 4.51 ^{C,e}	1071.87 ± 12.34 ^{A,d}	1494.79 ± 8.79 ^{B,c}	1753.32 ± 6.78 ^{B,b}	2364.66 ± 7.21 ^{A,b}	3596.79 ± 6.78 ^{B,a}
	C	101.54 ± 4.92 ^{C,f}	738.08 ± 4.31 ^{B,e}	757.697 ± 6.87 ^{B,e}	766.985 ± 9.75 ^{C,d}	1222.66 ± 3.67 ^{C,c}	1948.23 ± 5.67 ^{B,b}	3731.44 ± 4.67 ^{A,a}
	B	627.13 ± 5.83 ^{A,g}	1030.13 ± 3.42 ^{A,f}	1171.71 ± 4.56 ^{A,e}	2218.18 ± 9.2 ^{A,d}	2334.79 ± 3.45 ^{A,c}	2622.01 ± 4.21 ^{A,b}	3497.75 ± 4.53 ^{C,a}
Lactic acid	S	1240.41 ± 10.2 ^{A,e}	1280.23 ± 12.32 ^{A,d}	1493.23 ± 6.34 ^{A,d}	2157.74 ± 13.45 ^{A,d}	7006.16 ± 5.67 ^{A,c}	7897.14 ± 5.46 ^{A,b}	8946.28 ± 8.32 ^{A,a}
	C	574.58 ± 3.49 ^{C,g}	735.82 ± 6.71 ^{B,f}	1261.8 ± 10.1 ^{B,e}	1567.61 ± 10.21 ^{B,d}	2293.75 ± 3.50 ^{B,c}	2891.33 ± 5.32 ^{B,b}	6558.6 ± 6.78 ^{B,a}
	B	744.70 ± 9.01 ^{B,g}	1188.51 ± 9.23 ^{A,f}	1237.67 ± 13.21 ^{B,e}	1302.26 ± 8.79 ^{B,d}	2238.02 ± 4.56 ^{B,c}	2325.07 ± 9.01 ^{C,b}	2929.74 ± 5.76 ^{C,a}
Citric acid	S	26.41 ± 2.17 ^{B,d}	121.03 ± 7.37 ^{A,c}	140.87 ± 6.21 ^{A,b,c}	141.83 ± 5.21 ^{A,b}	164.09 ± 7.89 ^{A,b}	167.99 ± 3.21 ^{A,b}	250.32 ± 6.34 ^{B,a}
	C	72.79 ± 3.89 ^{A,f}	82.82 ± 3.21 ^{B,e}	84.45 ± 4.56 ^{B,e}	102.21 ± 4.67 ^{B,d}	127.56 ± 8.90 ^{B,c}	140.05 ± 4.51 ^{B,b}	153.28 ± 9.32 ^{A,a}
	B	26.14 ± 1.20 ^{B,e}	37.03 ± 4.56 ^{C,d}	41.52 ± 6.71 ^{C,c}	43.80 ± 5.21 ^{C,c}	55.18 ± 9.12 ^{C,b}	61.66 ± 3.42 ^{C,a}	65.95 ± 4.52 ^{C,a}
Formic acid	S	44.94 ± 3.45 ^{A,g}	86.41 ± 2.32 ^{A,f}	109.75 ± 3.46 ^{A,e}	143.27 ± 3.42 ^{A,d}	174.87 ± 5.67 ^{A,c}	206.70 ± 5.60 ^{A,b}	250.74 ± 3.45 ^{A,a}
	C	24.89 ± 2.13 ^{B,f}	74.01 ± 5.67 ^{A,e}	97.35 ± 5.21 ^{B,d}	134.24 ± 4.56 ^{A,c}	167.70 ± 3.46 ^{A,b}	172.99 ± 9.23 ^{B,b}	183.30 ± 4.56 ^{B,a}
	B	37.65 ± 2.46 ^{B,e}	38.04 ± 3.21 ^{B,e}	45.43 ± 4.89 ^{C,d}	52.04 ± 6.21 ^{B,c}	58.36 ± 3.21 ^{B,c}	71.35 ± 9.21 ^{C,b}	105.25 ± 5.21 ^{C,a}

In the table, B refers to the blank group with the basic MRS medium, S refers to the sample group with 15% soy protein hydrolysate (SPH) addition to MRS medium, C refers to the control group with 15% soy protein isolate (SPI) addition to MRS medium. Different uppercase letters and lowercase letters indicate significant differences between different groups at the same time and between sample groups, respectively, according to Tukey's test ($P < 0.05$).

obtained by supplementing permeate with whey protein hydrolysate was much higher than that with pure whey permeate (Fitzpatrick & O'Keeffe, 2001; Leh & Charles, 1989). Even during refrigerated storage, hydrogen peroxide and lactic acid could be produced by *L. delbrueckii* spp. *bulgaricus* with the supplement of casein hydrolysate (Sodini, Lucas, Oliveira, Remeuf, & Corrieu, 2002). Peptides can provide nitrogen source and promote bacterial metabolism such as producing organic acids, hydrogen peroxide and other substances. In this study, the addition of SPH increased the nitrogen content of the medium, which promoted the metabolism of *S. thermophilus*, producing a larger amount of organic acids and hydrogen peroxide (Table 3). The growth of *E. coli* was inhibited by hydrogen peroxide and organic acids. Therefore, the SPH addition could strengthen the antibacterial ability of *S. thermophilus*.

The results of our research showed that SPH addition to infant formula powder could significantly enhance the growth and metabolism of *Streptococcus thermophilus* and inhibit the growth of *Escherichia coli*. This study suggests that SPH may be a promising protein substituent for milk protein in infant formula powder.

4. Conclusions

All in all, adding 15%~25% soy protein hydrolysates (SPH) into infant milk powder could not only significantly stimulate *Streptococcus thermophilus* proliferation, but also inhibit the growth of *Escherichia coli* by producing organic acids and H₂O₂. Soy protein hydrolysates, containing low molecular weight peptide and less likely to cause an allergic reaction, would be a promising prebiotic and an alternative of cow's milk in the infant formula for the infants who are anaphylactic to cow's milk. However, the amino acid sequence compositions of the peptide in SPH and the mechanism of proliferation actions still need further studies.

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