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ORIGINAL RESEARCH PAPER

GENOTYPING CHARACTERIZATIONS ADN ANTIBIOTIC RESISTANCE OF *STREPTOCOCCUS THERMOPHILUS* STRAINS ISOLATED FROM DIFFERENT YOGURT BRANDS

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Abstract: This paper reported antibiotic resistance and PFGE genetic typing of *Streptococcus thermophilus* stains isolated from different yogurt brands in Chinese market. In total, 42 strains isolated from 43 yogurt samples were identified as *S. thermophilus* stains by 16S rDNA sequence analysis. The investigation of antibiotic-resistance profiles revealed that those isolates were resistant to erythromycin (69.05 %), clindamycin (73.81 %), chloramphenicol (61.90 %) and fosfomycin (19.05 %), and were susceptible to other 13 kinds of antibiotics. The genetic typing of *S. thermophilus* stains was examined by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA digested with SmaI. The enzyme restriction profiles showed the 42 *S. thermophilus* isolates were divided into 14 PFGE types, and further analysis showed the PFGE profile did not completely match with antibiotic resistance profile. The antibiotic resistance and PFGE pattern database generated in this study suggest that the safety evaluation of *S. thermophilus* should be paid more attention and will provide basic for information of food safe assessment of yogurt.

Keywords: *antibiotic resistance, genotyping, lactic acid bacteria, yogurt, 16SrDNA*

INTRODUCTION

Yogurt could be considered as a probiotic only if a probiotic strain has been used for its fermentation. *Streptococcus thermophilus* itself is not a probiotic species but certain strains were proven to be as such [1]. When *S. thermophilus* species is applied in producing yogurt, many advantages, such as producing flavor substances for adjusting flavor, reducing the time of milk curd, increasing viscosity, improving post acidification and playing probiotic effect, was found in previous reports [2 – 4]. In the past many years, lactic acid bacteria genera which were used in traditional fermented milk were generally considered as harmlessness for human being based on a long history of safe use [5]. The recent studies indicated that researcher should not ignore the safety evaluation involved probiotic lactic acid bacteria, and safety evaluation of the risk of drug resistance transfer was an important work [6]. If lactic acid bacteria have drug resistance, the resistance genes may be transmitted to the commensal bacteria or pathogenic bacteria in intestinal tract, thereby leading drug resistance metastasis and other serious consequences [6 – 9].

Bacterial typing is divided into two kinds of types (including phenotype and genotype), and drug resistance belong to phenotype. Pulsed-field gel electrophoresis (PFGE), which is a kind of typing method base on molecular level, have many advantages including high resolution, good repeatability, accurate results and accurate revealing subtle differences among genes in the huge genome [10]. At present, it is often used as the reference standard for judging the accuracy of other typing methods and has been recognized as the "gold standard" of typing methods for many years [11 – 13]. In recent years, PFGE technology has been developed and applied in dairy products for analyzing the roults of contamination of *L. monocytogenes* [14] and for evaluating *S. thermophilus* genotyping [15].

In Dong et *al.*'s study [16], only 15 isolates identified as *S. thermophilus* from retail yogurt was characterize genetically and was only evaluate the antimicrobial susceptibility profiles. However, there is still little comprehensive information involving drug resistance profile and PFGE profile of *S. thermophilus* strain isolated from yogurt. The present studies were based on two approaches. First, 42 strains isolated from yogurt were identified as *S. thermophilus* by 16SrDNA sequence analysis, and the drug resistance of those *S. thermophilus* strains was investigated according to the guidelines of the Clinical and Laboratory Standards Institute [17]. Second, those strains were genotyped by PFGE method to determine their electrophoretic karyotype. This approach allowed us to evaluate the types of *S. thermophilus* strains and its food safe used in preparing yogurt, and understand the relationship between the resistance and genomic DNA.

MATERIALS AND METHODS

Strains

123 strains were isolated from 43 yogurt samples in Chinese market. *Escherichia coli* ATCC 25922, *Streptococcus pneumoniae* ATCC 49619 and *Salmonella braenderup* H9812 were purchased from China National Institute for Drug and Biological Products.

16 SrDNA sequence analysis for those isolated strains

The method of extracting DNA from those isolated strains was referenced to reference book [18]. A pair of PCR primers including upstream primer (CTGGTCTGTAACTGACGCTGAG) and downstream primer (CCAACTGAATGATGGCAACTAA) was designed for 16 SrDNA sequence analysis. The conditions for 16S rDNA PCR were an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of a three-stage program with 30 S at 94 °C, 1 min at 61 °C for renaturation, then 1 min at 72 °C, and a final extension step for 10 min at 72 °C. The 16 SrDNA sequence was analysed, and then the sequencing results were compared with those registered in the Gene Bank database by BLAST analysis. Those results matched 16SrDNA sequence from *S. thermophilus* in the Gene Bank database were shown in Table 1.

Table 1. *Identification of S. thermophilus isolates by 16SrDNA analysis*

Strain number	16SrDNA identification		Strain number	16SrDNA identification	
	Result of identification	Matching rate [%]		Result of identification	Matching rate [%]
SR01	*S. thermophilus*	98	SR22	*S. thermophilus*	98
SR02	*S. thermophilus*	97	SR23	*S. thermophilus*	98
SR03	*S. thermophilus*	99	SR25	*S. thermophilus*	97
SR04	*S. thermophilus*	98	SR26	*S. thermophilus*	98
SR05	*S. thermophilus*	99	SR27	*S. thermophilus*	99
SR06	*S. thermophilus*	97	SR28	*S. thermophilus*	99
SR07	*S. thermophilus*	97	SR29	*S. thermophilus*	99
SR08	*S. thermophilus*	97	SR30	*S. thermophilus*	98
SR09	*S. thermophilus*	99	SR31	*S. thermophilus*	97
SR10	*S. thermophilus*	99	SR32	*S. thermophilus*	97
SR11	*S. thermophilus*	97	SR33	*S. thermophilus*	99
SR12	*S. thermophilus*	97	SR34	*S. thermophilus*	99
SR13	*S. thermophilus*	98	SR35	*S. thermophilus*	97
SR14	*S. thermophilus*	97	SR36	*S. thermophilus*	98
SR15	*S. thermophilus*	97	SR37	*S. thermophilus*	98
SR16	*S. thermophilus*	98	SR38	*S. thermophilus*	98
SR17	*S. thermophilus*	97	SR39	*S. thermophilus*	98
SR18	*S. thermophilus*	99	SR40	*S. thermophilus*	97
SR19	*S. thermophilus*	99	SR41	*S. thermophilus*	97
SR20	*S. thermophilus*	97	SR42	*S. thermophilus*	98
SR21	*S. thermophilus*	98	SR43	*S. thermophilus*	98

Antibiotic resistance analysis for those *S. thermophilus* isolates

Those strains cultured in Cation-adjusted Mueller–Hinton broth (The medium is composed of 17.5 $g \cdot L^{-1}$ casein acid hydrolysate, 3.0 $g \cdot L^{-1}$ beef extract and 1.5 $g \cdot L^{-1}$ starch, final *p*H value of 7.0） supplemented with lysed horse blood (2.5–5 %, V/V) as medium, and then their resistance test were performed by using the broth microdilution method of CLSI guidelines [18]. *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* ATCC 49619 were used as resistance quality control strains. The results were classified as susceptible (S), intermediate susceptible (I) and resistant (R) according to CLSI guidelines. Multidrug resistance (MDR) was defined as resistance to three or more groups of antimicrobial agents [19]. The types and concentrations of antibiotics and interpretive standard were shown in Table 2.

Table 2. *Antimicrobial agent and interpretive standard*

Number	Name	Abbreviation	Determined concentration [$\mu g \cdot mL^{-1}$]	MIC standard [$\mu g \cdot mL^{-1}$]		
				S(≤)	I	R(≥)
1	Ampicillin	AMP	0.03-32	8	16	32
2	Penicillin	PEN	0.03-32	8	16	32
3	Imipenem	IPM	0.002-2	0.5	1	2
4	Gentamicine	GEN	0.06-64	4	8	16
5	Vancomycine	VAN	0.25-256	4	8-16	32
6	Erythromycin	ERY	0.03-32	0.5	1-4	8
7	Clindamycin	CLI	0.015-16	0.5	1-2	4
8	Trimethoprin/ sulfamethoxazone	SXT	0.03-32	1	2	4
9	Amoxicillin/ clavulanic acid	AMC	0.03-32	2	4	8
10	Gatifloxacin	GAT	0.015-16	1	2	4
11	Chloramphenicol	CHL	0.015-16	1	2	4
12	Tetracycline	TET	0.06-64	2	4	8
13	Fosfomycin	FOS	0.25-256	8	16	32
14	Ceftriaxone	CRO	0.125-128	8	16	32
15	Cefotaxime	CTX	0.125-128	8	16	32
16	Rifampin	RIF	0.015-16	1	2	4

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis was performed according to the method described in Pulse-Net protocol [20]. DNA was digested with 30 unit of restriction enzyme SmaI at 37 °C. The restriction fragments were prepared by electrophoresis for 22 h at 14 °C in a CHEF Mapper system using pulsed times of 1–15 s. *S. Braenderup* H9812 was digested by using restriction enzyme SmaI for the DNA size marker. PFGE data were analyzed using GelCompar software (ver. 4.0; Applied Maths, Sint-Martens-Latem, Belgium). The extent of variability was determined by the Dice coefficient F, as previously described in previous literature [21]. Cluster construction was carried out by unweighted pair group average method (UPGMA) with a position tolerance of 0.10.

RESULTS AND DISCUSSION

The identification of isolated strains

The 42 strains among 123 isolates from yogurt brands were identified as *S. thermophilus* by using 16 SrDNA technology, and the matching rates (over 96 %) were very high，as shown in Table 2. Biochemical identification also had carried out in the study (the data was not shown) and the results were identical with those obtained by 16 SrDNA analysis. Subsequently, the 42 strains were used in the following study.

Drug resistance of *S. thermophilus* strains

The results of antibiotic resistance of all *S. thermophilus* strains were shown in Table 3. 42 strains were resistant to 4 antibiotics (ERY, CHL, FOS and RIF), and the resistance rates were 69.05 %, 61.90 %, 73.81 % and 19.05 %, respectively. The intermediate susceptible degree were observed for ERY, CLI, CHL and FOS, and intermediate susceptible rates were 21.43 %, 7.14 %, 19.05 % and 14.29 %, respectively. 13 antibiotic display more than 50 % sensitivity rates. The sensitive rates to 11 kinds of antibiotics (AMP, PEN, IPM, GEN, VAN, SXT, AMC, GAT, TET, CRO and CTX) among 13 antibiotics were 100 %, and sensitive rates to CLI and RIF observed for *S. thermophilus* strains were 92.86 % and 80.95 %.

Table 3. *Antibiotic resistance of 42 S. thermophilus isolates*

Strains number	Resistant spectrum	Strains number	Resistant spectrum
SR02	ERY-CHL-FOS-RIF	SR13	ERY-FOS
SR18	ERY-CHL-FOS-RIF	SR30	ERY-FOS
SR01	ERY-CHL-FOS	SR35	ERY-FOS
SR16	ERY-CHL-FOS	SR38	ERY-FOS
SR17	ERY-CHL-FOS	SR23	ERY
SR04	ERY-CHL-FOS	SR29	ERY
SR05	ERY-CHL-FOS	SR33	ERY
SR06	ERY-CHL-FOS	SR42	ERY
SR19	ERY-CHL-FOS	SR25	CHL-FOS-RIF
SR26	ERY-CHL-FOS	SR39	CHL-FOS-RIF
SR28	ERY-CHL-FOS	SR41	CHL-FOS-RIF
SR22	ERY-CHL-FOS	SR08	CHL-FOS
SR32	ERY-CHL-FOS	SR12	CHL-FOS
SR34	ERY-CHL-FOS	SR15	CHL-FOS
SR21	ERY-CHL-RIF	SR31	CHL-FOS
SR36	ERY-CHL-RIF	SR07	CHL
SR40	ERY-CHL	SR27	FOS
SR14	ERY-CHL	SR09	FOS
SR20	ERY-FOS-RIF	SR10	FOS
SR03	ERY-FOS	SR37	/
SR11	ERY-FOS	SR43	/

Resistance spectrum of 42 strains can be seen from Table 4. Two strains (SR37 and SR43) were not resistant to all antibiotics, 20 strains displayed multi drug resistant, and the remaining 20 strains which were resistant to 1 or 2 antibiotics was not multi drug resistant. The result revealed that some strains had similarity resistance spectrum, and they were mainly resistant to ERY, CHL and FOS.

Table 4. *Antibiotic resistance of 42 S. thermophilus isolates*

Strain number	Types of antibiotic															
	AMP	PEN	IPM	GEN	VAN	ERY	CLI	SXT	AMC	GAT	CHL	TET	FOS	CRO	CTX	RIF
SR01	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR02	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	R
SR03	S	S	S	S	S	R	S	S	S	S	I	S	R	S	S	S
SR04	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR05	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR06	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR07	S	S	S	S	S	I	S	S	S	S	R	S	I	S	S	S
SR08	S	S	S	S	S	I	S	S	S	S	R	S	R	S	S	S
SR09	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
SR10	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
SR11	S	S	S	S	S	R	S	S	S	S	I	S	R	S	S	S
SR12	S	S	S	S	S	I	S	S	S	S	R	S	R	S	S	S
SR13	S	S	S	S	S	R	S	S	S	S	I	S	R	S	S	S
SR14	S	S	S	S	S	R	S	S	S	S	R	S	I	S	S	S
SR15	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S
SR16	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR17	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR18	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	R
SR19	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR20	S	S	S	S	S	R	S	S	S	S	I	S	R	S	S	R
SR21	S	S	S	S	S	R	S	S	S	S	R	S	I	S	S	R
SR22	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR23	S	S	S	S	S	R	S	S	S	S	I	S	S	S	S	S
SR25	S	S	S	S	S	I	S	S	S	S	R	S	R	S	S	R
SR26	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR27	S	S	S	S	S	I	S	S	S	S	I	S	R	S	S	S
SR28	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR29	S	S	S	S	S	R	S	S	S	S	I	S	I	S	S	S
SR30	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S
SR31	S	S	S	S	S	I	I	S	S	S	R	S	R	S	S	S
SR32	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR33	S	S	S	S	S	R	S	S	S	S	I	S	S	S	S	S
SR34	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S	S
SR35	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S
SR36	S	S	S	S	S	R	S	S	S	S	R	S	I	S	S	R
SR37	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR38	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S
SR39	S	S	S	S	S	I	S	S	S	S	R	S	R	S	S	R
SR40	S	S	S	S	S	R	I	S	S	S	R	S	I	S	S	S

SR41	S	S	S	S	S	I	S	S	S	S	R	S	R	S	S	R
SR42	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
SR43	S	S	S	S	S	I	I	S	S	S	S	S	S	S	S	S
R [%]	0.00	0.00	0.00	0.00	0.00	69.05	0.00	0.00	0.00	0.00	61.90	0.00	73.81	0.00	0.00	19.05
I [%]	0.00	0.00	0.00	0.00	0.00	21.43	7.14	0.00	0.00	0.00	19.05	0.00	14.29	0.00	0.00	0.00
S [%]	100.00	100.00	100.00	100.00	100.00	9.52	92.86	100.00	100.00	100.00	19.05	100.00	11.90	100.00	100.00	80.95
ATCC 49619	S	S	S	\	S	S	S	S	S	S	S	S	S	S	S	S
ATCC 25922	\	\	\	S	\	\	\	\	\	\	\	\	\	\	\	\

Note: R, Resistant; S, Susceptible; I, Intermediate susceptible; \, No Detection

Dong et *al*. result [16] displayed only 1 isolate among 15 *S. thermophilus* strains was more resistant to ERY. Aslim & Beyatl [22] reported that most *S. thermophilus* strains obtained from various villages in different regions of Turkey had resistance to GEN and PEN and was sensitive to CHL and TET. In *Zhou* et *al.* study [23], the resistance to AMP, TET, CHL and GEN and sensitivity to PEN were observed among *S. thermophilus* strains collected from dairy plants located in different places in China. Those studies indicated that the *S. thermophilus* strains obtained from yogurt brands had difference in antibiotic resistance compared to those of other studies. Multiple antibiotic resistances of many *S. thermophilus* strains in the study were revealed and maybe related to the drug resistance gene, such as msrC, vanX, and dfrA [24]. Those results reminded us that antibiotic resistance of *S. thermophilus* strains should been paid more attention.

PFGE typing of *S. thermophilus* strains

The results of cluster analysis of the 42 *S. thermophilus* strains by using PFGE method were shown in Fig.1. Those strains were divided into 14 PFGE types, and H type included 13 strains isolated from 3 yogurt brands. 7 strains were clustered to F type (those strains were isolated from three yogurt brands) and I type（those strains were isolated from two yogurt brands. A type, B type, D type and L type, which each contained 2 strains, were isolated from 2, 1, 1 and 2 yogurt brands, respectively. C type, E type, G type, J type and K type each contained one strain, and each was isolated from one yogurt brand. Each of the M and N types included one strain and belonged to the same yogurt brand. Erkus et al isolated 66 *S. thermophilus* strains from artisanal Yuruk yogurts and obtained 22 homology groups by using PFGE analysis [25]. Our result in the study and Erkus et al result suggested *S. thermophilus* strains have rich diversity of PFGE genotyping.

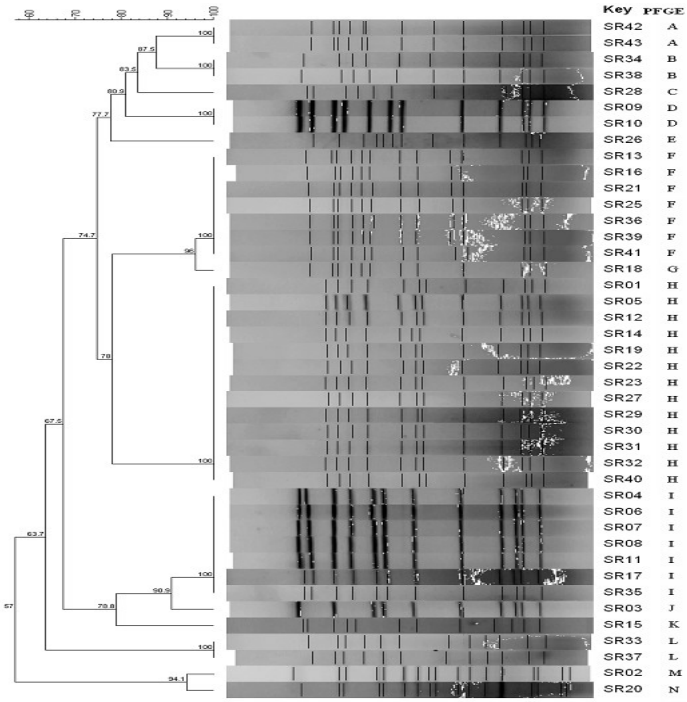

Figure 1. *Dendrogram of 42 S. thermophilus isolates digested with SmaI studied by PFGE*

Pulsed field gel electrophoresis is a typing method that digests the bacterial chromosome DNA by using rare restriction endonuclease sites for obtaining the size of 5-30 chromosome fragment about 10-800 kb, and then separated DNA fragments of different sizes by electrophoresis [26 – 28]. The technology can show subtle changes in genomes and have many advantages such as good stability, high resolution, and not disturbance from the mutability of phenotypic traits, so it is very important to understand the genetic characteristics of bacteria [29]. In the study, 42 *S. thermophilus* strains isolated from different yogurt brands in Chinese market were divided into 14 PFGE types. The results suggested that *S. thermophilus* used in producing yogurt in Chinese market may be different strains and may be from different strain suppers. The results, which the strain with same PFGE types was applied in different yogurt brands, also suggested that those closely genetic related strains were used in producing different yogurt brands. The most probable reason was those yogurt factories hardly developed new strain used in producing yogurt by themselves and only purchased from designated bacterial producing enterprise.

There is no evidence in present result proving the correlation between the antibiotic resistance pattern and the PFGE profile, as could be found in Table 5. Some strains with different PFGE typing displayed the same drug resistance spectrums. The strains among the same PFGE typing had different antibiotic resistance patterns, likely due to mutations of resistance genes, but the mutation site was not in the position of the restriction enzyme site. Consequently, the status of antibiotic resistance of *S. thermophilus* strains could not been evaluated by PFGE genotyping.

Table 5. *Comparison between resistance spectrum and PFGE profiles of 42 S. thermophilus isolates*

Strain number	Resistance pattern	PFGE profiles	Strain number	Resistance pattern	PFGE profiles
SR02	ERY-CHL-FOS-RIF	M	SR13	ERY-FOS	F
SR18	ERY-CHL-FOS-RIF	G	SR30	ERY-FOS	H
SR01	ERY-CHL-FOS	H	SR35	ERY-FOS	I
SR16	ERY-CHL-FOS	F	SR38	ERY-FOS	B
SR17	ERY-CHL-FOS	I	SR23	ERY	H
SR04	ERY-CHL-FOS	I	SR29	ERY	H
SR05	ERY-CHL-FOS	H	SR33	ERY	L
SR06	ERY-CHL-FOS	I	SR42	ERY	A
SR19	ERY-CHL-FOS	H	SR25	CHL-FOS-RIF	F
SR26	ERY-CHL-FOS	E	SR39	CHL-FOS-RIF	F
SR28	ERY-CHL-FOS	C	SR41	CHL-FOS-RIF	F
SR22	ERY-CHL-FOS	H	SR08	CHL-FOS	I
SR32	ERY-CHL-FOS	H	SR12	CHL-FOS	H
SR34	ERY-CHL-FOS	B	SR15	CHL-FOS	K
SR21	ERY-CHL-RIF	F	SR31	CHL-FOS	H
SR36	ERY-CHL-RIF	F	SR07	CHL	I
SR40	ERY-CHL	H	SR27	FOS	H
SR14	ERY-CHL	H	SR09	FOS	D
SR20	ERY-FOS-RIF	N	SR10	FOS	D
SR03	ERY-FOS	J	SR37	/	L
SR11	ERY-FOS	I	SR43	/	A

As we known, there is a litter study of *S. thermophilus* involving resistance spectrum and PFGE profile in a report. Although Dong et *al.* report [16] evaluated the resistance spectrum and PFGE profile of *S. thermophilus*, only 15 isolates of *S. thermophilus* was used in the analysis of resistance spectrum and PFGE profile, and the resistance to only six kinds of antibiotics for those *S. thermophilus* strains was evaluated. Thus, the present study can help us deepen understanding in food safety of *S. thermophilus.*

CONCLUSIONS

In summary, the study constructed antibiotic resistance profile and PFGE genotype profile of 42 *S. thermophilus* isolated from Chinese yogurt products. The results revealed that all *S. thermophilus* strains had resistance to ERY, CHL, FOS and RIF. PFGE genotype profile was clustered into 14 homology groups. The possible provided sources of 42 *S. thermophilus* strains were preliminarily analyzed. The strains with same PFGE typing did not display the same drug resistance spectrum. These findings will help us to evaluate and control food safety risk of those *S. thermophilus* strains used in preparing yogurts.

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