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ORIGINAL RESEARCH PAPER

Mild heat stress limited the post-acidification caused by *Lactobacillus rhamnosus* hsryfm 1301 in fermented milk

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Abstract
Objective Fermented milk is the optimal vehicle for delivering probiotic bacteria. However, the viable count of probiotic bacteria such as some lactic acid bacteria and the post-acidification of fermented milk are a contradiction. The objective of this study was to restrict the post-acidification of the fermented milk containing living *Lactobacillus rhamnosus* hsryfm 1301.
Results Mild heat stress treatment (46 °C, 1 h) was chosen to help control the post-acidification caused by *L. rhamnosus* hsryfm 1301. When fermented milk was produced by single *L. rhamnosus* hsryfm 1301, the heat stress treatment reduced the post-acidification from 0.39 to 0.11% lactic acid, and the viable cells were maintained above 2.0×10^8 CFU mL^{-1} during 21 days of storage. Although the post-acidification limitation of heat treatment was relatively weak in fermented milk produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02 (from 0.26 to 0.10% lactic acid), this treatment was still effective. Furthermore, no whey separation in the fermented milk was caused by the treatment.
Conclusions Mild heat stress treatment could limit the post-acidification caused by *L. rhamnosus* hsryfm 1301 by decreasing its metabolism and proliferation. This treatment is a promising strategy to improve the shelf life of probiotic fermented milk.

Keywords Post-acidification limitation · Probiotics · Proliferation · Shelf life · Stress treatment

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Introduction

Probiotic bacteria have been increasingly proposed as health promoting bacteria in a variety of food system, because of their safety, functional, and technological characteristics (Kanmani et al. 2013). *Lactobacillus rhamnosus* has been one of the most widely studied and used probiotic species because of its abundance of probiotic properties, such as adjusting intestinal flora, regulating immune function, protecting intestinal epithelial cells, reducing blood lipids, and decreasing dental caries and respiratory tract infections (Chen et al. 2014; de Almada et al. 2015; Segers and Lebeer 2014; Tannock et al. 2000; Wickens et al. 2012).

It is recommended that a probiotic product should contain at least 10^6 CFU g^{-1} of viable probiotic cells throughout the entire shelf life of the product to ensure their health-promoting effects (Settachaimongkon et al. 2016). Milk, which is a natural medium for probiotics, such as some lactic acid bacteria (LAB), is the optimal vehicle for delivering *L. rhamnosus* strains that are able to utilize lactose (Marco et al. 2017). However, a large number of living probiotics easily cause post-acidification in functional yogurts.

Several strategies, such as pasteurization, strain mutagenesis (Jaichumjai et al. 2010), and supplementing antibacterial substances (Bali et al. 2016), have been developed to control the post-acidification caused by traditional starters. However, these strategies are imperfect with respect to probiotic strains. Pasteurization is inappropriate for functional yogurts, and the use of antibacterial substances are tightly restricted in many countries. Mutagenesis is inefficient with respect to probiotic strains because it is necessary to give consideration to both the probiotic properties and the fermentation characteristics of the mutants. Post-acidification control strategies are intended to weaken the metabolic activity of the starters during storage to decrease the accumulation of lactic acid. Mild stresses can slow the metabolism of probiotics, but not kill the bacteria, and mild stresses can also transform some of the bacteria into paraprobiotics (de Almada et al. 2016; Papadimitriou et al. 2016). Sometimes, sublethal stress conditions can even protect probiotics from a harsher environment (Settachaimongkon et al. 2015). Therefore, correct stress treatments may help control the post-acidification of functional yogurts. Among the common stresses, the heat stress is easy to implement during processing and does not introduce anything to dairy products. A previous study by the authors showed that heat stress at 46 °C for 1 h enhanced the aerotolerance of *L. rhamnosus* hsryfm 1301 (Zhang et al. 2018) and did not reduce its viable count.

In the present study, it was investigated that whether mild heat stress treatment could help control the post-acidification of functional fermented milks containing *L. rhamnosus* hsryfm 1301.

Materials and methods

Bacterial strain and growth conditions

L. rhamnosus hsryfm 1301 possesses valuable probiotic properties such as adjusting intestinal flora and reducing blood lipids (Chen et al. 2014). *L. rhamnosus* hsryfm 1301 was isolated from the gut of subjects from Bama, Guangxi Province, China. *Streptococcus thermophilus* grx02 was isolated from a traditional dairy product from Xinjiang, China. Both of these strains were preserved in the Jiangsu Provincial Key Lab of Dairy Biotechnology and Safety Control, Yangzhou University. *L. rhamnosus* hsryfm 1301 was refreshed in MRS broth (2% (v/v) inoculation) at 37 °C for 24 h under static incubation. *S. thermophilus* grx02 was refreshed in LM17 broth (M17 medium supplemented 0.5% lactose, 2% (v/v) inoculation) at 42 °C for 12 h under static incubation.

Preparation of the fermented milk

Preparation of skim milk: Reconstituted milk (10% skim milk powder) was added to 7% sucrose and homogenized for 15 min (20 MPa, 60 °C). The samples were then heated at 105 °C for 5 min. Preparation of the strains: *L. rhamnosus* hsryfm 1301 was incubated in MRS for 24 h and then in skim milk for 16 h at 37 °C; *S. thermophilus* grx02 was incubated in LM17 for 12 h and then in skim milk for 12 h at 42 °C.

All the fermented milk samples were prepared in test tubes (inner diameter: 1.5 cm). Fermented milk produced by single *L. rhamnosus* hsryfm 1301 was prepared as follow. The prepared *L. rhamnosus* hsryfm 1301 was added to skim milk to a final concentration of 2% (v/v) inoculum and cultured at 37 °C until the milk coagulated (approximately 14 h). Fermented milk containing *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02 was prepared by adding the strains to skim milk to a final concentration of 2% (v/v) inoculum and cultured at 37 °C until the milk coagulated (approximately 6 h). The fermentation was performed in three replicates for each type of fermented milk.

Mild heat stress treatment

The heat stress treatment was set according to the result that heat stress at 46 °C could enhance the aerotolerance of *L. rhamnosus* hsryfm 1301 (Zhang et al. 2018). Control group: the prepared fermented milk samples were stored at 4 °C. Treatment group: the prepared fermented milk samples were treated at 46 °C for 1 h and then stored at 4 °C.

Determination of acidification profile

Production of acid during storage was expressed by changes in pH and increases in titratable acidity (TA). The pH measurements were performed using a laboratory pH meter (Mettler Toledo FE20, Shanghai, China). The TA value of fermented milk samples was assessed following the Association of Analytical Communities (AOAC) titration method: 10 g fermented milk sample was mixed with 20 mL water and then were titrated with 0.1 M NaOH by using phenolphthalein as the indicator. The TA value was expressed as a percentage of lactic acid, determined using the following equation (Nguyen et al. 2014):

$$\%\text{lactic acid} = V_{NaOH} \times 0.09/10$$

where V_{NaOH} is the volume (mL) of 0.1 M NaOH solution required for titration.

Determination of colony forming units (CFU)

CFU were calculated with a previously described drop plate technique (Sieuwerts et al. 2008). Fermented milk samples were serially diluted 10-fold, and the dilution time (T) of the original sample was 0. For dilution, 5-μL samples were pipetted onto a plate containing 1.5% agar medium. The plates were air-dried and incubated until the colonies were visible, with an average colony size of 200 to 500 μm. The colony number of every drop (N_{colony}) was then counted. LM17 agar at 42 °C for 20 h was used for *S. thermophilus* grx02. *L. rhamnosus* hsryfm 1301 was enumerated using glycoprival MRS agar at 37 °C for 48 h (Zhang et al. 2019).

The number of CFU per milliliter was calculated using the following equation:

$$\text{CFU mL}^{-1} = 10^{T} \times 200 \times N_{colony}$$

The lower limit of detection (LLOD) was 200 CFU mL^{-1}.

Statistical analysis

Significant differences between two values were evaluated by unpaired Student *t* test ($p < 0.05$).

Results

Acidification profile of the fermented milk during storage

The changes in pH and TA (TA was expressed as % lactic acid) reflected the coincident acidification of each sample group. The TA of the fermented milk produced by *L. rhamnosus* hsryfm 1301 was 0.62 (pH 5.02) before storage and consistently increased to 1.01 (pH 4.35) in 15 days (Fig. 1a). This result was a notable postacidification. While the TA of the heat-treated fermented milk produced by *L. rhamnosus* hsryfm 1301 reached 0.66 (pH 4.92) before storage and increased to just 0.77 (pH 4.87) in 15 days (Fig. 1a). The heat stress treatment suspended the acidification of the fermented milk in the first 3 days and reduced the post-acidification by 70% ($p < 0.05$). Interestingly, the TA of both the control and treatment samples declined slightly in the last 6 days (Fig. 1a).

The changes in pH and TA were also investigated in fermented milk produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02 because these 2 species are often used together (Jia et al. 2016; Kort et al. 2015). The TA of the mixed strains fermented milk increased from 0.73 (pH 4.85) to 1.00 (pH 4.46) in 6 days (Fig. 1b). After treated at 46 °C for 1 h, the TA of the mixed strains fermented milk reached 0.84 (pH 4.62) before storage and increased to just 0.93 (pH 4.53) in 15 days (Fig. 1b). Heat stress treatment slowed and reduced the post-acidification of the mixed strain fermented milk. After 15 days of storage, the decrease in TA also occurred in the mixed strains fermented milk samples, regardless of whether they were heat-treated (Fig. 1b).

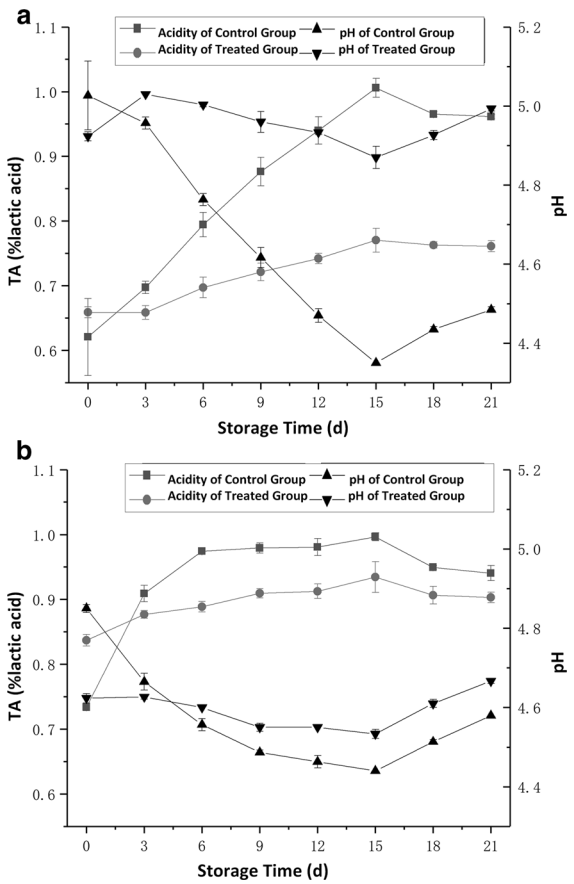
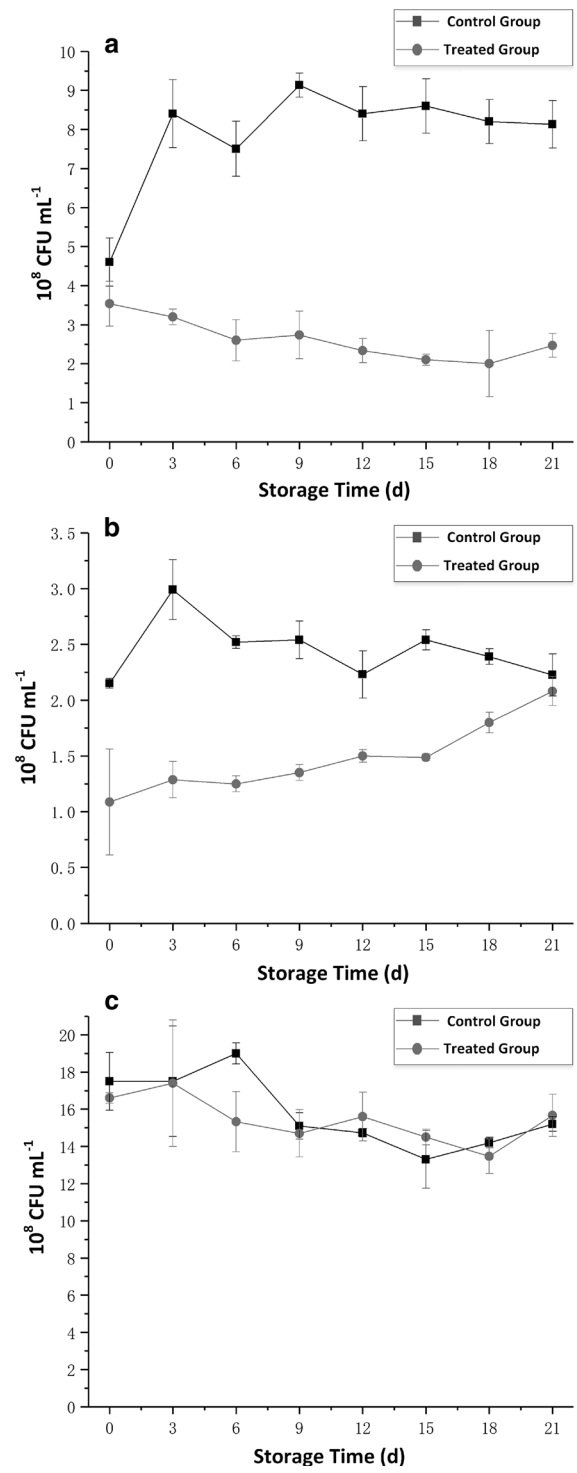


Fig. 1 TA and pH of the heat-treated samples. **a** Fermented milk samples were produced by *L. rhamnosus* hsryfm 1301. **b** Fermented milk samples were produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02. The control group samples were stored at 4 °C, and the treated group samples were treated at 46 °C for 1 h before stored

Viable counts of the starters in the fermented milk during storage

In the fermented milk produced by single *L. rhamnosus* hsryfm 1301, the viable count of *L. rhamnosus* hsryfm 1301 was 4.6×10^8 CFU mL^{-1} before storage. This count increased to 8.4×10^8 CFU mL^{-1} in 3 days and remained nearly constant over the next 18 days ($p > 0.05$). The heat stress treatment decrease the CFU to 3.5×10^8 mL^{-1}, but the CFU was maintained above 2.0×10^8 CFU mL^{-1} during the last 15 days ($p > 0.05$) (Fig. 2a).

In the fermented milk produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02, the living count of *L. rhamnosus* hsryfm 1301 was 2.2×10^8 - CFU mL^{-1} before storage, which was lower than that



of the single strain fermented milk. This count increased to 3.0×10^8 CFU mL^{-1} in 3 days and decreased slowly over the next 18 days. The heat

◀**Fig. 2** Viable counts of the heat-treated samples. **a** Viable counts of *L. rhamnosus* hsryfm 1301 in the fermented milk samples produced by *L. rhamnosus* hsryfm 1301. **b** Viable counts of *L. rhamnosus* hsryfm 1301 in the fermented milk samples produced by *Lactobacillus rhamnosus* hsryfm 1301 and *S. thermophilus* grx02. **c** Viable counts of *S. thermophilus* grx02 in the fermented milk samples produced by *Lactobacillus rhamnosus* hsryfm 1301 and *S. thermophilus* grx02. The control group samples were stored at 4 °C, and the treated group samples were treated at 46 °C for 1 h before stored

stress treatment decreased the CFU of *L. rhamnosus* hsryfm 1301 to 1.1×10^8 mL^{-1}, but the CFU increased to 2.1×10^8 CFU mL^{-1} during the whole storage process (Fig. 2b). The living count of *S. thermophilus* grx02 was sustained between $1.3–1.9 \times 10^9$ CFU mL^{-1} during the storage process, regardless of whether the samples were heat-treated (Fig. 2c).

Whey separation

Whey separation is one of the most common problems during yogurt storage, and heat treatment may facilitate this problem (Chandan and O'Rell 2006). So, the whey separation of the heat-treated samples was investigated. No whey separation was observed in the treated fermented milk produced by *L. rhamnosus* hsryfm 1301 or *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02 during 21 days of storage (Fig. 3). The fermented milk samples did not flow when they were positioned at an angle of 60°.

Discussion

Probiotics are living microorganisms and, when administered in adequate amounts, confer a beneficial health effect to the host. In recent years, probiotics have been used as treatments of various diseases (Kanmani et al. 2013). To date, most of the probiotic bacteria comprise of the genera of LAB. It is pleasing to consume LAB probiotics in delicious food. Fermented milk is the optimal vehicle in delivering most probiotics (Marco et al. 2017). However, in most instances, the living count of probiotics and the post-acidification of the fermented milk are a contradiction. The viable cell number and sensory are both very important for probiotic fermented milk. To control the post-acidification, a factor that weakens the metabolic activity of probiotics strains, but does not kill them, is needed during storage. In the present study, mild heat stress treatment (46 °C, 1 h) was chosen to help control the post-acidification.

Since the starters were still growing when the samples were heat-treated, the TA of the treated samples was higher than that of the control samples. However, after treatment, the post-acidification of the samples decreased, resulting in lower TA than control samples. Interestingly, the TA of all the samples decreased slightly during the last 6 days, which might be due to the pH homeostasis enzymes such as glutamate decarboxylase and arginine deiminase (Jaichumjai et al. 2010).

In the fermented milk produced by single *L. rhamnosus* hsryfm 1301, 24% of the cells were killed by the mild heat stress treatment. This result was different from the result measured in MRS that heat stress at 46 °C could enhance the stress tolerance of *L. rhamnosus* hsryfm 1301 (Zhang et al. 2018). This difference might result from the influence of the media and pH differences on the heat tolerance of *L. rhamnosus* hsryfm 1301. But the cell death did not continue, and the viable cells were maintained above 2.0×10^8 CFU mL^{-1} during 21 days of storage. The CFU of *L. rhamnosus* hsryfm 1301 in 100 mL of this fermented milk could reach 10^{10}, which is far above the dosage benefiting human health ($10^8–10^9$ CFU) (Jayamanne and Adams 2006). When exposed to a sudden upshift in temperature causing protein denaturation, living cells increase the expression of heat shock proteins (HSPs) to ensure survival, which also slows down cell metabolism and proliferation (Varmanen and Savijoki 2011). The *L. rhamnosus* hsryfm 1301 cells almost went into metabolic arrest after heat-treated, and as a result, the final TA was just 86°T during storage. This result would improve the shelf life of products significantly.

The use of *S. thermophilus* as starters provides additional benefits to the final product, such as controlled whey separation and improved viscosity, flavor, consistency and color. Therefore, *L. rhamnosus* and *S. thermophilus* are often used together (Jia et al. 2016; Kort et al. 2015). In the fermented milk produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02, the viable count of *L. rhamnosus* hsryfm 1301 was relatively low for the short incubation time. The cell death of *L. rhamnosus* hsryfm 1301

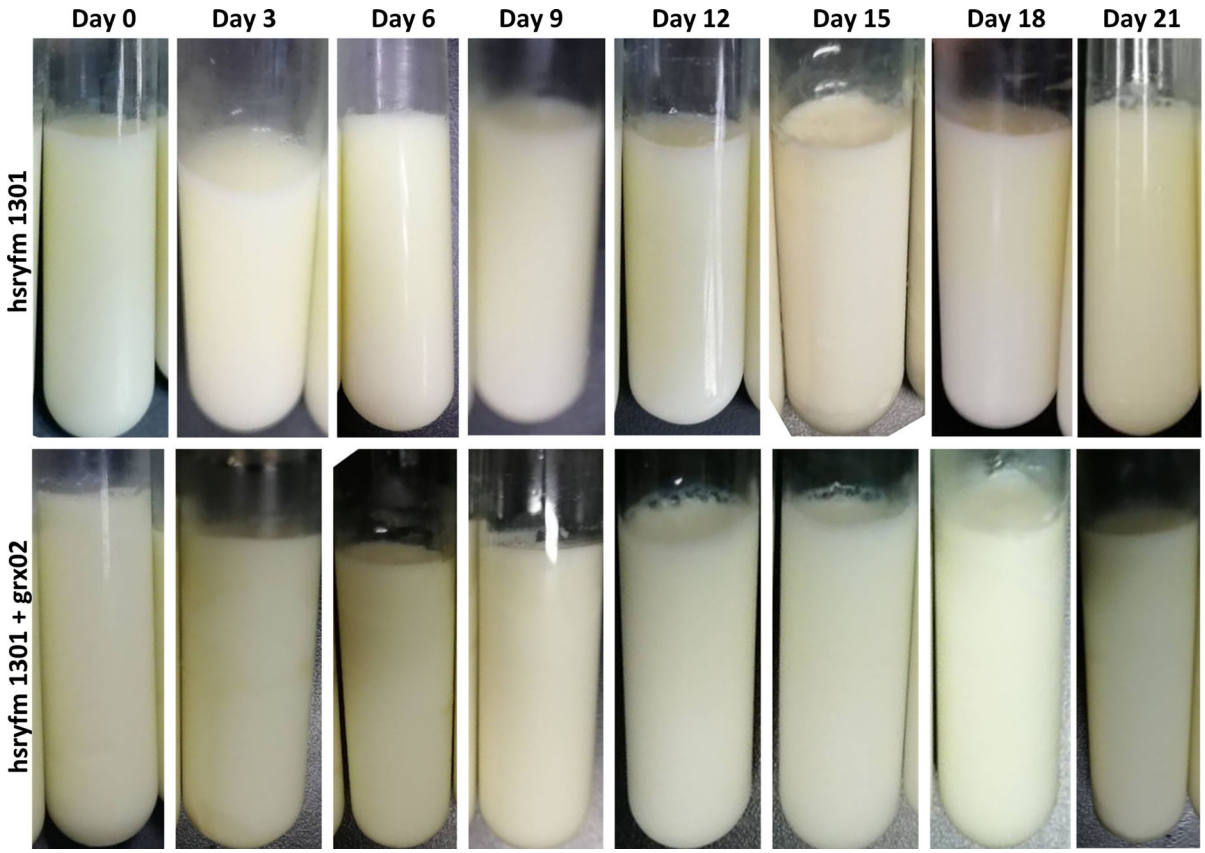


Fig. 3 Photographs of the heat-treated samples. The samples were treated at 46 °C for 1 h before stored

was more striking than that in the single strain fermented milk, which might result from the lower pH. However, the viable count of *L. rhamnosus* hsryfm 1301 increased over the storage process, which was equivalent to that of the single strain fermented milk at the end of storage. However, *S. thermophilus* grx02 had higher heat tolerance than *L. rhamnosus* hsryfm 1301, and its viable count was not influenced by the heat treatment. Therefore, the limitation of post-acidification by heat treatment was weaker in the fermented milk produced by *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02. LAB share many similarities in the mechanism of heat tolerance (Papadimitriou et al. 2016), so the post-acidification limitation of heat treatment can possibly work in probiotic fermented milk produced by other LAB.

Whey separation can be facilitated by heat treatment (Chandan and O'Rell 2006). Fortunately, the temperature used in this study was higher than the production temperature of common yogurt by only 4 °C. No whey separation was observed in the treated fermented milk produced by *L. rhamnosus* hsryfm 1301 or *L. rhamnosus* hsryfm 1301 and *S. thermophilus* grx02 during 21 days of storage.

In conclusion, mild heat stress treatment (46 °C, 1 h) could limit the post-acidification caused by *L. rhamnosus* hsryfm 1301 by decreasing its metabolism and proliferation. At the same time, the viable count of *L. rhamnosus* hsryfm 1301 remained at a high level. Mild heat stress treatment is a promising strategy to improve the shelf life of probiotic fermented milk.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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