

# Influence of Different Sterilization Conditions on the Growth and Exopolysaccharide of *Streptococcus thermophilus* and Co-Cultivation with *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1

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## Abstract

The sterilization conditions between experiment and dairy industrial level are different and concern about influence on product's qualities. In this study, the fermentation properties of *Streptococcus thermophilus*, alone and in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus*, were evaluated in skim milk that had been subjected to distinct sterilization conditions. Growth, organic acid generation, and EPS production were determined using pasteurized or autoclaved milk. When *S. thermophilus* was cultivated in pasteurized skim milk, the growth was strain-dependent. On the other hand, growth of *S. thermophilus* was accelerated in autoclaved milk. Exocellular polysaccharide (EPS) production by *L. bulgaricus* was not affected by the combination of *S. thermophilus* strains. Thus, we observed that yogurt fermented by *L. bulgaricus* was minimally affected by the combination of *S. thermophilus* strains; growth of *L. bulgaricus* was maintained under the constant environment. These results should facilitate the development of fermented milk produced from *L. bulgaricus* in the dairy industry.

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## Keywords

***Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, Sterilization, Formic Acid, Exopolysaccharide**

## 1. Introduction

The international standards of the WHO/FAO define yogurt as milk fermented with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, and production of yogurt depends on a symbiosis established between the two species [1]. The combination of both species is very important for fermented milk production. In this symbiosis, it has been demonstrated that formic acid, folic acid [2], pyruvic acid, and carbon dioxide [3] [4] are generated by *S. thermophilus* during an early phase; subsequently, peptides and amino acids are generated by *L. bulgaricus*. Formic acid has been shown to be an essential element for bacterial growth, because formic acid produced by *S. thermophilus* is used as precursor of the purine bases required for DNA replication and cell proliferation by *L. bulgaricus* [5] [6]. In the case of dairy products, some formic acid is generated upon heat treatment of milk; thus, abiotic formation of endogenous formic acid (during sterilization of the milk substrate) contributes to the growth of yogurt starters. However, we are not aware of reports regarding the formation of formic acid during sterilization of skim milk, which is usually performed at the laboratory level.

Both *S. thermophilus* and *L. bulgaricus* are able to produce lactic acid by homo-fermentation of carbohydrates. However, it has been found that *Streptococcus* genus possesses Pyruvate Formate-Lyase (PFL) and is able to produce formic acid in glycolysis. Namely, *S. thermophilus* can't produce just lactic acid, or formic acid in aerobic condition. The organic acid profile of bacteria has been shown to change according to metabolic activity [7] [8]. Although carbohydrate metabolism and organic acid production by *S. thermophilus* are very important in fermented milk manufacture, there have been few reports on the fermentation patterns of these strains [9]-[10].

We have been investigating the use of probiotic strain *L. bulgaricus* OLL1073R-1 as a commercial yogurt starter. This strain produces exocellular polysaccharide (EPS), and the physiology of host immunological response has been elucidated [12]-[16]. In previous work, we demonstrated that cell viability and EPS production by *L. bulgaricus* OLL1073R-1 were enhanced by supplementation of the medium with formic acid, and were commended by culturing of OLL1073R-1 in combination with *S. thermophilus* able to produce high levels of formic acid [17]. In the present study, we examined the growth and organic acid production of *S. thermophilus*, and evaluated the influence of co-cultivation with *S. thermophilus* on EPS production by *L. bulgaricus* OLL1073R-1.

## 2. Materials and Methods

### 2.1. Strains and Cultures

*Streptococcus thermophilus* NIAI510 was obtained from National Institute of Animal Industry. *Streptococcus thermophilus* ME-551 and *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 were obtained from Meiji Co., Ltd. (Tokyo, Japan). The strains were propagated twice in autoclaved (115°C, 15 min) 10% (w/v) skim milk. The cultures were inoculated into pasteurized (63°C, 30 min) or autoclaved 10% skim milk and incubated at 37°C for 24 hr.

### 2.2. Growth of *S. thermophilus* in the Different Media

The strains were propagated twice in autoclaved 10% skim milk and inoculated at 1% (v/v) to pasteurized or autoclaved skim milk. The tubes were incubated at 37°C. After incubation for 3, 6, 9, 12, or 24 hr, cultures were examined for pH and total cell numbers. The pH was recorded with a pH meter (pH/ION meter F-24, HORIBA, Tokyo, Japan). Total cell numbers were determined using optical densities (ODs) at 600 nm. In the case of skim milk culture, ODs were determined after mixing one volume of culture with nine volumes of a solution composed of 0.2% (w/v) NaOH and 0.2% sodium EDTA according to the method of Sander *et al.* (2010). ODs were monitored using a UV-mini1240 spectrophotometer (Shimazu Co., Kyoto, Japan).

### 2.3. Organic Acid Analysis in Skim Milk Cultures

After 24 hr of incubation, analyze of organic acids in the culture was outsourced to the Kyoto Institute of Nutrition and Pathology Inc. (Kyoto, Japan). Levels of succinic acid, lactic acid, formic acid, and acetic acid were determined by ion-exclusion chromatography using HPLC. The amounts of each organic acid were estimated by comparing retention times and peak areas to those obtained using standards.

### 2.4. EPS Determination

The concentrations of EPS in the cultures were determined as described elsewhere [17]. Briefly, EPS obtained using an ultra-filter unit (USY-1, MW 10,000 cut-off, ADVANTEC Co. Ltd., Tokyo, Japan) was washed with distilled water and the amount of the residue on the filter was determined as neutral sugars using the phenol-H<sub>2</sub>SO<sub>4</sub> method [18].

### 2.5. Viable Cell Numbers of *S. thermophilus* and *L. bulgaricus* in the Co-Culture

The strains, which had been cultivated in autoclaved skim milk, were inoculated at 2% (v/v) into pasteurized or autoclaved skim milk. The tubes were incubated for 24 hr at 37°C. The samples then were serially diluted ten-fold using sterile physiological saline (0.85% NaCl (w/v)) to quantify the cell numbers of both strains. Diluted solutions (100 µl) of the co-cultures were spread on MRS agar and M17 agar plates for detection of *L. bulgaricus* and *S. thermophilus*, respectively. The plates were incubated at 37°C for 48 hr under anaerobic condition with Anaero Pack (Sugiyama-Gen, Co., Ltd., Tokyo, Japan) and the colony forming units (CFU/ml) were enumerated.

### 2.6. Statistics

Statistical significance in differences was determined using the Student's *t*-test. The test was performed as two-tailed and P value was considered statistically significant difference less than 0.05.

## 3. Results

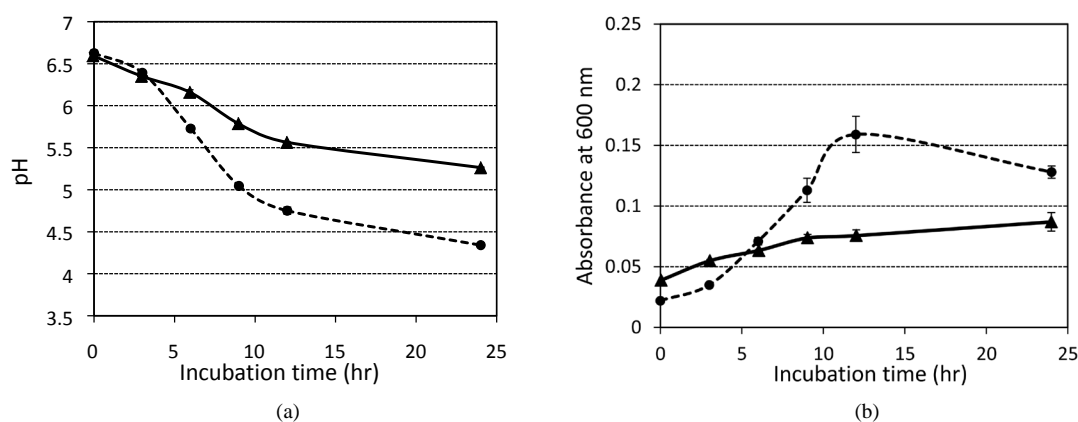
### 3.1. Influence of Sterilization Condition and Organic Acid Production of Skim Milk in the Skim Milk Cultures

We determined acidification and turbidity in skim milk of *S. thermophilus* strains in order to understand their fermentation pattern in substrates subjected to different sterilization conditions. The growth of the two *S. thermophilus* strains differed in skim milk sterilized by pasteurization, and the difference was starting at 6 hr after inoculation (**Figure 1**). Notably, NIAI510 grew more rapidly than ME-551 in pasteurized skim milk. However, the patterns of acidification and turbidity of both strains in autoclaved milk were very similar (**Figure 2**). In both substrates, the turbidity of the NIAI510 culture was decreased slightly from 12 to 24 hr incubation.

It is well known that formic acid generated by *S. thermophilus* is essential for the growth of *L. bulgaricus*. We detected organic acids generated by fermentation (**Table 1**). After 24 hr of incubation in the pasteurized milk, formic acid was completely consumed in mixed and mono-culture of *L. bulgaricus*. We estimated that both *S. thermophilus* strains produced formic acid at 0.2 to 0.25 mM during 24 hr culturing in skim milk, while *L. bulgaricus* required formic acid at concentrations of at least 0.2 mM to support optimal growth in skim milk over the same interval. In autoclaved milk, the organic acid profile was altered; notably, autoclaving of skim milk resulted in the (abiotic) generation of formic acid to concentrations of approximately 2 mM. Consumption of formic acid (in autoclaved milk) by co-cultured *S. thermophilus* and *L. bulgaricus* differed depending on the strain of *S. thermophilus* used. Furthermore, we observed that *S. thermophilus* ME-551 generated not only formic acid but also succinic acid. Mono-culture of *L. bulgaricus* OLL1073R-1 in pasteurized milk yielded succinic and acetic acids at higher levels.

### 3.2. Influence of the Sterilization Condition of Skim Milk on Growth of the Co-Culture

To investigate the influence of mixed culture with *S. thermophilus* and *L. bulgaricus* in skim milk treated under different conditions, *i.e.* pasteurized or autoclaved sterilization, viable cell numbers were counted (**Table 2**).



**Figure 1.** Culture kinetics of *S. thermophilus* growing on pasteurized 10% skim milk. Strains were grown at 37°C. pH (a) and optical density (b) of *S. thermophilus* strains in 10% skim milk are shown. Filled circle (●) and filled triangle (▲) symbols indicate the data points for strain NIAI510 and ME-551, respectively. Optical density was monitored at 600 nm. Values were indicated means triplicate within a single experiment. Error bars were intended as standard deviation.

**Table 1.** The amounts of organic acids in the mono-cultures or co-cultures of *S. thermophilus* and *L. bulgaricus*.

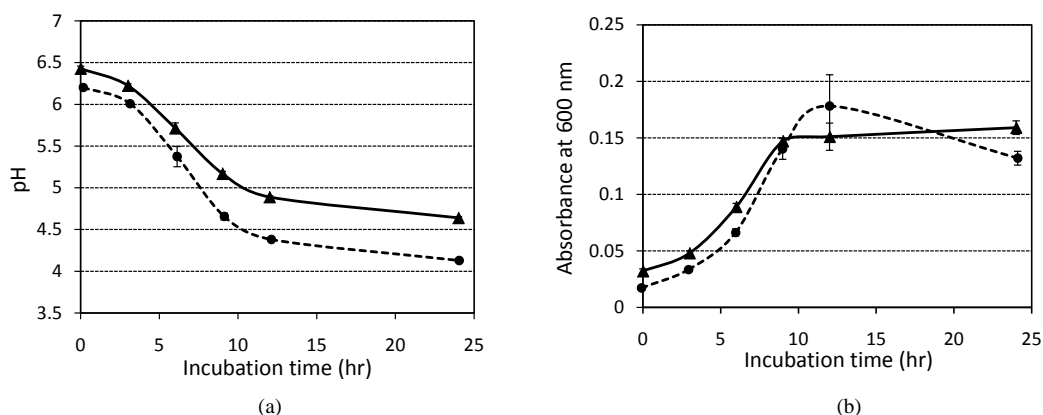
|  | Succinic acid | Lactic acid | Formic acid | Acetic acid |
|--|---------------|-------------|-------------|-------------|
| <u>Pasteurized skim milk (63°C, 30 min)</u>                      |               |             |             |             |
| Control  | 0.085         | 0.079       | 0.014       | 0.000       |
| <i>S. thermophilus</i> NIAI510                                   | 0.038         | 64.511      | 0.201       | 0.278       |
| <i>S. thermophilus</i> ME-551                                    | 0.089         | 40.420      | 0.253       | 0.363       |
| <i>L. bulgaricus</i> OLL1073R-1                                  | 0.981         | 63.263      | 0.000       | 1.974       |
| <i>L. bulgaricus</i> OLL1073R-1 + <i>S. thermophilus</i> NIAI510 | 0.853         | 78.752      | 0.000       | 1.691       |
| <i>L. bulgaricus</i> OLL1073R-1 + <i>S. thermophilus</i> ME-551  | 0.651         | 82.391      | 0.000       | 1.795       |
| <u>Autoclaved skim milk (115°C, 15 min)</u>                      |               |             |             |             |
| Control  | 0.088         | 0.000       | 2.101       | 0.142       |
| <i>S. thermophilus</i> NIAI510                                   | 0.037         | 64.553      | 1.247       | 0.319       |
| <i>S. thermophilus</i> ME-551                                    | 0.126         | 55.124      | 3.083       | 0.856       |
| <i>L. bulgaricus</i> OLL1073R-1                                  | 0.445         | 77.593      | 0.597       | 1.744       |
| <i>L. bulgaricus</i> OLL1073R-1 + <i>S. thermophilus</i> NIAI510 | 0.697         | 83.162      | 0.316       | 1.870       |
| <i>L. bulgaricus</i> OLL1073R-1 + <i>S. thermophilus</i> ME-551  | 0.568         | 81.998      | 0.549       | 1.841       |

The values are showed the concentration of each organic acid as mM.

**Table 2.** Cell viability of *L. bulgaricus* and *S. thermophilus* in the skim milk sterilized by different conditions.

|                      | Pasteurized skim milk<br>(63°C, 30 min) (CFU/ml) | Autoclaved skim milk<br>(115°C, 15 min) (CFU/ml) |
|----------------------|--|--|
| OLL1073R-1           | $9.87 \times 10^8$                               | $4.20 \times 10^9$                               |
| OLL1073R-1 + ME-551  |  |  |
| OLL1073R-1           | $4.04 \times 10^9$                               | $4.03 \times 10^9$                               |
| ME-551               | $7.93 \times 10^7$                               | $<10^7$  |
| OLL1073R-1 + NIAI510 |  |  |
| OLL1073R-1           | $2.10 \times 10^9$                               | $6.27 \times 10^9$                               |
| NIAI510              | $1.82 \times 10^8$                               | $<10^7$  |

The data shows the mean of three plates.



**Figure 2.** Culture kinetics of *S. thermophilus* on autoclaved 10% skim milk. Strains were grown at 37°C. pH (a) and optical density (b) of *S. thermophilus* strains in autoclaved 10% skim milk are shown. Filled circle (●) and filled triangle (▲) symbols indicate the data points for strain NIAI510 and ME-551, respectively. Optical density was monitored at 600 nm. Values were indicated means triplicate within a single experiment. Error bars were intended as standard deviation.

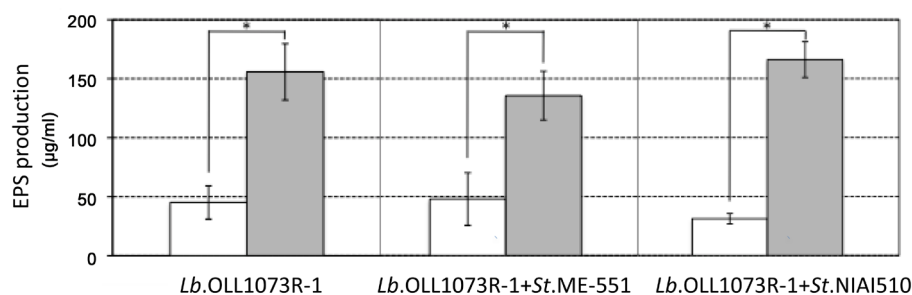
After co-cultivation for 24 hr in pasteurized milk, cell proliferation of *L. bulgaricus* was stimulated and *L. bulgaricus* cell numbers increased (compared to mono-culture) four- or two-fold upon co-culturing with *S. thermophilus* ME-551 or NIAI510, respectively. When *L. bulgaricus* was cultivated with *S. thermophilus* in autoclaved milk, the *L. bulgaricus* cell numbers also were enhanced. However, under these conditions, no *S. thermophilus* colonies were detected by replica plating, meaning that cell numbers fell below  $10^7$  CFU/ml (the limit of detection in our assay).

### 3.3. Influence on EPS Production by *L. bulgaricus* OLL1073R-1 of Different Sterilization Conditions and Co-Cultivation with *S. thermophilus*

*L. bulgaricus* OLL1073R-1 is a starter used for the manufacture of yogurt and is hypothesized to confer benefits on the host by producing EPS. To investigate influence of the medium sterilization and co-culturing on EPS production by *L. bulgaricus* OLL1073R-1, the amount of EPS generated by *L. bulgaricus* OLL1073R-1 was determined on skim milk subjected to different sterilization conditions and co-cultivated with *S. thermophilus* (Figure 3). The 24 hr EPS yields in the autoclaved or pasteurized skim milk co-cultures were 136 - 166 µg/ml and 31 - 48 µg/ml, respectively. Thus, an approximately 4-fold increase in EPS production was detected with autoclaved milk compared to pasteurized milk. It was found that positive correlation was confirmed with EPS production and the sterilization condition of skim milk in Student's t-test ( $p < 0.01$ ). The level of EPS production by *L. bulgaricus* did differ depending on the co-cultivated *S. thermophilus* strain used.

## 4. Discussion

The sterilization condition of milk can differ greatly between experimental and manufacturing environments. Industrial sterilization has been designed to preclude the denaturation of milk proteins as much as possible. On the other hand, milk-based medium in laboratory usually is sterilized by autoclaving at 110°C - 115°C for 10 to 15 min. The pressure on these conditions were about 1.3 atm (110°C) and 1.6 atm (115°C), it have been considered that milk components after autoclaving are degraded by both temperature and pressure. Thus, laboratory-derived experimental data may not be directly applicable for manufacturing purposes. In the present study, we investigated the difference between pasteurized and autoclaved skim milk as a medium for growth of yogurt starter cultures. In our hands, two different *S. thermophilus* strains yielded similar growth patterns when cultured in autoclaved skim milk, whereas the two strains yielded distinct growth patterns when cultured in pasteurized skim milk. And our tests revealed that the levels of lactic and formic acids in skim milk were higher following autoclaving (113.3°C, 10 min); previous work demonstrated that propionic, pyruvic, butyric, and acetic acids also were increased following autoclaving [19]. A previous report indicated that autoclaving of infant formula leads to decreased concentrations of total protein, free amino acid, and amino acids, and the accumulation of ammonia [20]. Based on these findings, we speculate that the elevated levels of organic acids (such as formic acid) and amino acids generated by autoclaving of skim milk affected the growth of *S. thermophilus*.



**Figure 3.** Comparison of EPS concentrations after 24 hr cultivation in skim milk sterilized by different processes. EPS was prepared from skim milk sterilized by pasteurization (□) or autoclaving (■). Values are presented as the mean  $\pm$  standard deviation of eight samples. These experiments were replicated within a single experiment. Significant differences were calculated by Student's t-test ( $p$ : \* < 0.01).

*S. thermophilus* and *L. bulgaricus* are widely used as LAB in the dairy industry, and these species are known to form a symbiosis. To elucidate the cooperative growth of these species, we investigated the fermentation properties of *S. thermophilus* strains, focusing on the generation of organic acids. We observed that two separate *S. thermophilus* strains (ME-551 and NIAI510) generated 0.2 - 0.25 mM formic acid and 0.3 - 0.4 mM acetic acid in the course of 24 hr of growth in pasteurized milk; neither of the tested strains produced detectable levels of succinic acid in cultures growing on pasteurized milk. In autoclaved skim milk, ME-551 was able to produce organic acids to higher levels than was NIAI510. In a previous report, *Streptococcus mutans* was shown to produce higher levels of formic acid and lower levels of lactic acid under anaerobic conditions [21]. In addition, it was demonstrated that the fermentation of glycerol by *Escherichia coli* in a low-supplement medium under anaerobic conditions led to the generation of ethanol, succinic acid, acetic acid, and formic acid, whereas lactic acid was not detected in the extracellular medium [22]. Based on these findings, we speculate that formic acid generation from pyruvic acid on glycolysis more efficient in ME-551 than that in NIAI510 under anaerobic conditions, suggesting that ME-551 is more suitable for the production of fermented milk than is NIAI510.

Qualitative analysis of organic acids in *L. bulgaricus* OLL1073R-1 mono-culture and in co-culture with *S. thermophilus* also was performed. Succinic and acetic acid levels were higher in the mono-culture of *L. bulgaricus* OLL1073R-1 on pasteurized milk compared to that on autoclaved milk; in particular, the concentration of succinic acid was remarkably increased on pasteurized milk. It has been recognized that *L. bulgaricus* is one kind of homofermentative lactic acid bacteria, in which only lactic acid is generated from pyruvic acid on glycolysis. On the other hand, hetero-fermentation is able to produce acetic acid and ethanol other than lactic acid via hexose monophosphate pathway. Especially, acetic acid is generated from acetyl-phosphate. In *Lactobacillus plantarum*, succinic acid is generated from phosphoenol pyruvic acid, pyruvic acid, and acetyl CoA via the TCA cycle, especially under anaerobic condition [23]. In the present study, acetic acid might be produced via an alternative pathway; we speculate that compounds generated by autoclaving might alter carbohydrate metabolism by *L. bulgaricus*.

Furthermore, *L. bulgaricus* cell numbers exceeded those of *S. thermophilus* after co-cultivation for 24 hr. Previous reports indicated that the viable cell numbers of *S. thermophilus* in yogurt were higher than those of *L. bulgaricus* [15] [16] [24], and bacterial cell numbers of *L. bulgaricus* in yogurt were increased by stirring during fermentation [25]. Based on these findings, we hypothesize that bacterial cell numbers of *L. bulgaricus* and *S. thermophilus* in yogurt depend on cultivation time and environmental conditions. Namely, the *S. thermophilus* bacterial population is expected to be higher in cultures grown for shorter times or under anaerobic conditions, whereas *L. bulgaricus* numbers are expected to be higher in cultures grown for longer times or under aerobic conditions. In the present study, the use of autoclaved milk as a substrate appeared to favor the growth of *L. bulgaricus*.

In addition, we investigated the effect of co-culturing with *S. thermophilus* on EPS production by *L. bulgaricus* OLL1073R-1. We showed that EPS production was influenced by the sterilization condition used, and did not differ for the two different *S. thermophilus* strains used for co-cultivation. In our previous study, we demonstrated that *L. bulgaricus* OLL1073R-1 cultivated in formate-containing medium produced EPS at levels four-fold greater than those of a control [17]. In the present study, the EPS yield after co-cultivation with *S. thermophilus* in pasteurized milk was similar to that seen with formate supplementation in the previous study, suggest-



ing that the substrate (milk) sterilization condition had a larger effect than the co-culturing with *S. thermophilus*. At the same time, we found that the EPS production ability of *L. bulgaricus* OLL1073R-1 was very stable.

Thus, we have demonstrated that the process used for sterilization of milk for yogurt manufacture has large effects on the growth of the starter cultures, the level of functional EPS production, and the required time of fermentation. These data will facilitate improved economics for the dairy food industry.

## 5. Conclusion

The influences of different sterilization conditions of milk against dairy products have not been investigated. The present study demonstrated the growth of *S. thermophilus* and *L. bulgaricus*, and EPS production by *L. bulgaricus*, when grown on milk subjected to different sterilization processes. We showed that the growth and organic acid generation or consumption by both strains varied according to sterilization conditions. In addition, the yield of EPS produced from *L. bulgaricus* was influenced by sterilization condition rather than the specific identity of the co-cultured *S. thermophilus* strain. Our results are expected to provide fundamental data for use by the dairy industry.

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