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EVALUATION OF THE EFFICACY OF E-POLYLYSINE AGAINST NUTRITIONAL MICROBES IN YOGURT

Dina N. Ali* and Huda Elsayed

Reference Lab for Safety Analysis of Food of Animal Origin, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.

***Corresponding Author: Dina N. Ali**
Reference Lab for Safety Analysis of Food of Animal Origin, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.

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ABSTRACT

Being a natural antibacterial cationic peptide, ε-polylysine (ε-PL) is applied as a food preservative, however, the mechanism through which it acts against some food pathogens in yogurt has not been elucidated. This study focused on the inhibitory effect of ε-PL at different concentrations (0.005, 0.015 and 0.025%) against *S.aureus, L.monocytogenes* and *E.coli* inoculated in laboratory made yogurt. The most active functional groups were detected in ε-PL using Fourier-transform infrared spectroscopy (FTIR). The peptide could inhibit all the above mentioned pathogens two days after inoculation during refrigerated storage at 0.025%. Sensory properties was evaluated and it was revealed that ε-PL was the most preferred preservative by consumers with respect to taste, color, appearance and overall acceptability. As green consumerism is becoming more popular, there is a growing demand for natural preservatives. This study provides basic theories and methods for the application of ε-PL against food-borne pathogens, as well as scientific guidelines for consumers and producers in the agricultural sector. The results obtained may promote the utilization of ε-PL in the food industry, reduce the use of chemical preservatives and increase food safety.

KEYWORDS: *S.aureus; L.monocytogenes; E.coli;* FTIR; consumer; sensory evaluation.

INTRODUCTION

Fermentation is one of the earliest processes used by humans to transform milk into products with a longer storage duration or shelf- life. Although yogurt has many health benefits, it has a serious drawback in that it can be contaminated by a variety of pathogens during manufacturing and storage, especially if hygiene is not strictly enforced (Rajapaksha et al., 2013). Microorganisms that cause food poisoning cause more than one billion gastrointestinal tract inflammations each year, with an estimate of 5 to 13 million deaths. The most common food pathogens namely *Escherichia.coli, Staphylococcus.aureus*, and *Listeria.monocytogenes*, cause a variety of diseases in humans (FDA, 2010; Elsherif and Ali, 2020).

Natural antimicrobial compounds have been extensively researched and analyzed in recent decades for their potential application as food preservatives and medications (Gopinath et al., 2022).

Biosynthesized ε -PL is a water – soluble, non - covalent linear homopoly-amino acid with an ionic strength of about 9.0. It is a peptide bond between carboxyl groups and -amino groups of L-lysine residues rather than the standard peptide bonds joining ε –PL (Mark et al., 2020). This peptide generated by a membrane-bound ε -PL synthetase with non-ribosomal peptide synthetase properties and it does not covalently bind to the elongating ε -PL chain during polymerization. As it decomposes into lysine in the human body, ε-PL is a useful source of this amino acid and, because of its biodegradability, it is also an edible, nontoxic, and environmentally benign dietary element (Dodd et al., 2018). In October 2004, the Food and Drug Administration (FDA) approved ε -PL as a safe food preservative (FDA, 2004). A reasonably high concentration of ε -PL (20,000 µg/g) was administered perorally via food to rats, and the results revealed no evident histological alterations or carcinogenicity (Hosomi et al., 2015). It has also been established that ε -PL does not produce harmful effect in terms of reproduction, neurology, immunology, or embryo and fetus development (Zhang et al., 2020).

Only few published studies are available on this subject, all of which investigated the inhibitory concentrations of ε -PL against bacteria in culture broth media, despite its promising antimicrobial characteristics. Many of these studies reported that 0.0001% to 0.01% of ε-PL inhibited the growth of various Gram-positive and Gram-negative bacteria, including, among others, *S. aureus, Micrococcus spp., Bacillus spp., Clostridium acetobutylicium, Campylobacter jejuni, Salmonella.*

typhimurium, E. coli, Pseudomonas spp., and various lactic acid bacteria (Shima et al., 1984; Hiraki et al., 2003 and Shih et al., 2006). More recently, the antimicrobial activity of ε-PL against food-borne pathogens such as *L. monocytogenes, E. coli,* and *S. typhimurium* alone, as well as in combination with lactic acid, acetic acid, sodium diacetate, or sodium lactate has also been widely reported in culture broth but it has not been studied in food systems (Dahong et al., 2022).

As the published information on ε-PL's antimicrobial activity is limited to broth culture media, the aim of this study was to determine the most active functional groups of ε-PL by FTIR and the effects of this natural compound against *E.coli, S. aureus,* and *L. monocytogenes* in food systems, by inoculating the pathogens and ε-PL at different concentrations in yogurt stored at refrigerated temperature.

MATERIALS AND METHODS

Bacterial suspension preparation

The bacterial strains examined in this study were *E. coli* (ATCC 9637), *S. aureus* (ATCC 29213), and *L. monocytogens* (NCTC 13372\ ATCC® 7644), and they were obtained from Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.

The strains were pre-enriched on selective broth and selective agar, and this procedure was specifically carried out based on AOAC protocols (2001) for *S. aureus*, ISO (11290-2:2017) for *L. monocytogenes*, and on BAM, (2022) for *E. coli.* The bacterial suspension using a 0.5 McFarland Standard according to McFarland (1907).

Characterization of ε-PL powder by FTIR

Fourier-transform infrared spectroscopy (FTIR, NICOLET, iS10, Thermo Scientific) was conducted at the Chemistry Department at the Faculty of Science, Assiut University, and the potassium bromide pellet method was used for sample preparation. Nujol mulls, and then the samples, were scanned in the FTIR spectrometer at a wavenumber range of 4000–500 cm^{-1} (Rozenberg and Shoham, 2007; Elsherif and Al Shrief, 2021).

Challenge of ε-PL's antibacterial activity in manufactured yogurt

Raw milk was boiled for 10 min then suddenly cold and was then inoculated with 2% yogurt culture at 45°C. One ml of each strain suspension (prepared as indicated in section 2.1) was mixed with 100 ml of prepared milk and was dispensed into suitable sterile jars. Then, ε-PL was added at concentrations 0.005, 0.015, and 0.025%, while an additional jar without ε-PL was used as positive control, and the jars were incubated at 40°C until curdling. The jars, including the control jar (free from bacterial suspension and ε-PL as a negative control), were stored at refrigeration temperature (4 ± 2°C). The inoculated jars were examined bacteriologically for the count of *S. aureus* using Baird-Parker media, *L. monocytogenes* using ALOA agar plates, and *E. coli* using SMAC agar (37°C for 24−48 h) after curdling (at time zero) and periodically, until the experiment ended.

Sensory evaluation of manufactured yogurt

Yogurt was prepared as previously mentioned and was divided into four equal portions; each was subjected to the previous treatments (without adding any previous reference pathogens). Samples were stored at refrigeration temperature (4 ± 2°C). Twenty-three consumers were selected and grouped based on age, sex, and education to taste the samples. The perception of consumers toward the yogurt prepared using various treatments was studied with respect to three different attributes: odor, flavor, texture and overall acceptability (OAA).

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5.04 (GraphPad, Inc., San Diego, USA) and Statistical 12.0 (Dell, Inc., Tulsa, USA). Least significant differences were determined at $p < 0.05$. The data were represented using Microsoft Excel spreadsheets.

RESULTS

The Ftir analysis of ε-PL revealed the presence of many peaks from 3442.96, 3008.58, 2925.64, and 2854.53 cm^{-1}. Sharp differences in peaks were detected at the end in Fig. 1 as 1746.60, 1636.78, 1465.55, 1377.86, 1241.54, 1156.08, 723, 580.72 and end with 469.52 cm^{-1}.

The efficacy of ε-PL against the most common food-borne pathogens namely, *E. coli, S. aureus,* and *L. monocytogens* was evaluated and is presented in Fig. 2. The peptide reduced the count of *E. coli* from 5 to 1 log_{10} on the fourth day after yogurt inoculation and refrigerated storage at concentrations of 0.015 and 0.025%, but the bacterium could be detected until the sixth day with ε-PL at 0.005%, and hardly any change in bacterial count was observed in the control. In contrast, *S. aureus* and *L. monocytogens* were reduced from 5 log_{10} to complete inhibition on the second day after yogurt storage at an ε-PL concentration of 0.025% and were observed until the fourth day at 0.015%, but were inhibited on the sixth day of storage with ε-PL at 0.005%, with little reduction observed in the control.

The organoleptic properties of ε-PL are presented in Fig. 3. The ε-PL at different concentrations have no significant different with control one but more favorable than the last with odor and texture 98% for control, 0.05, and 0.015% of ε-PL and 97% for 0.025% of ε-PL. The flavor of control yogurt was 95% while 97% for ε-PL at 0.005% with OAA 97% and 96% for ε-PL at 0.015 and 0.005%.

FIGURE CAPTIONS

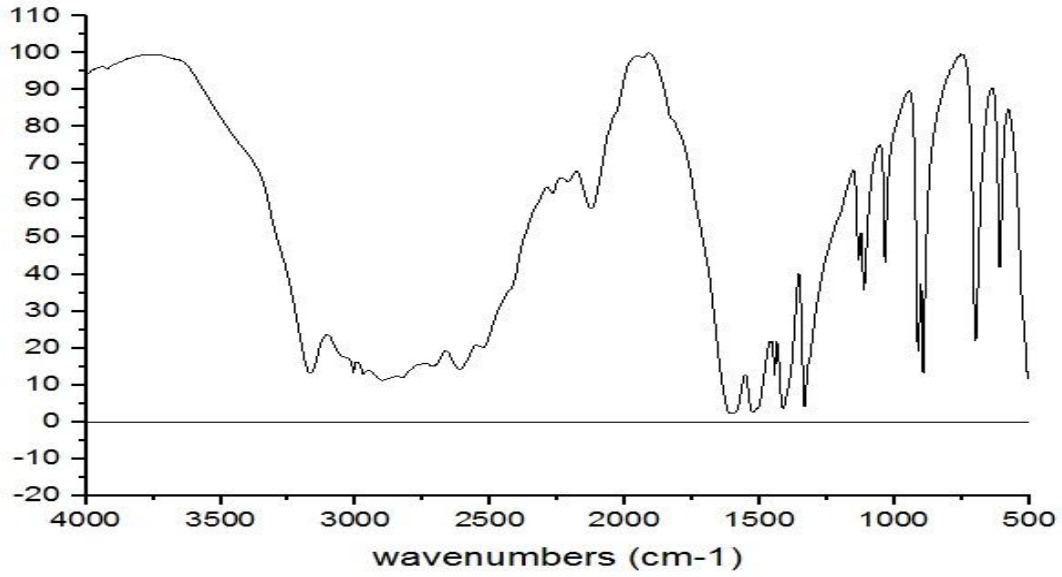
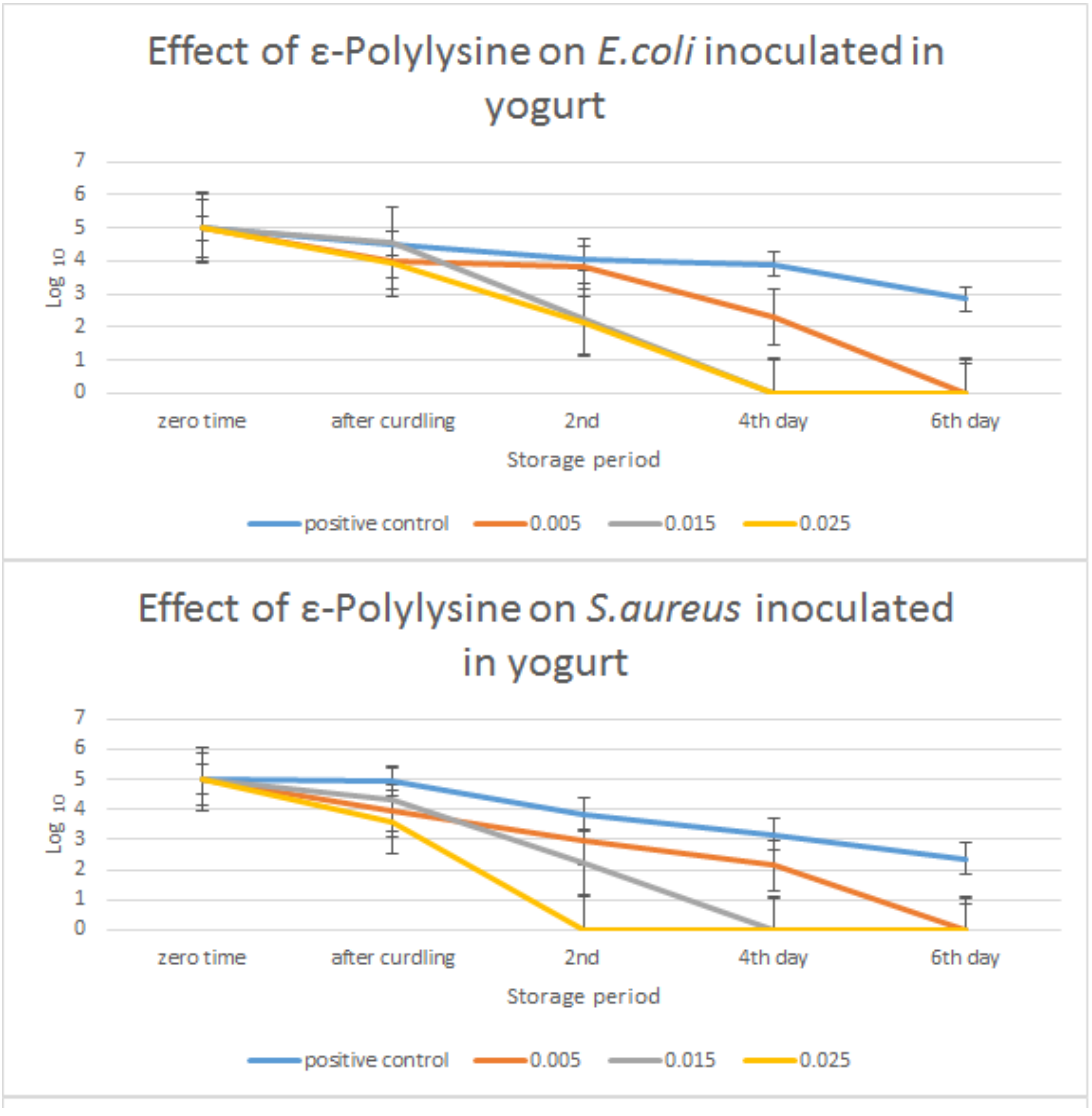


Fig. 1: FTIR of ε-Polylysine.



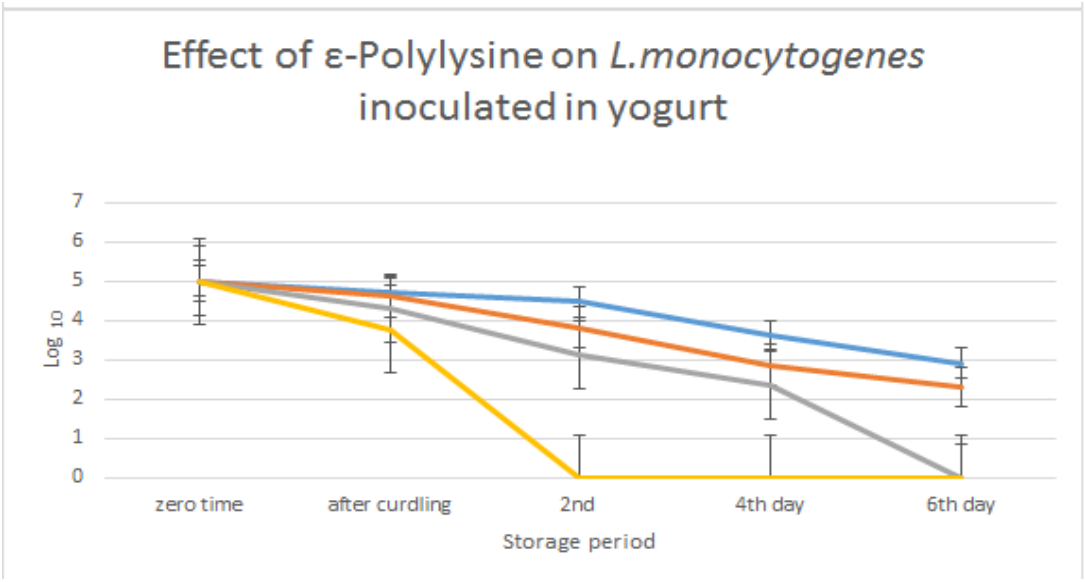


Fig. 2: Effect of different concentrations of ε-Polylysine on different inoculated food-borne pathogens in refrigerated yogurt.

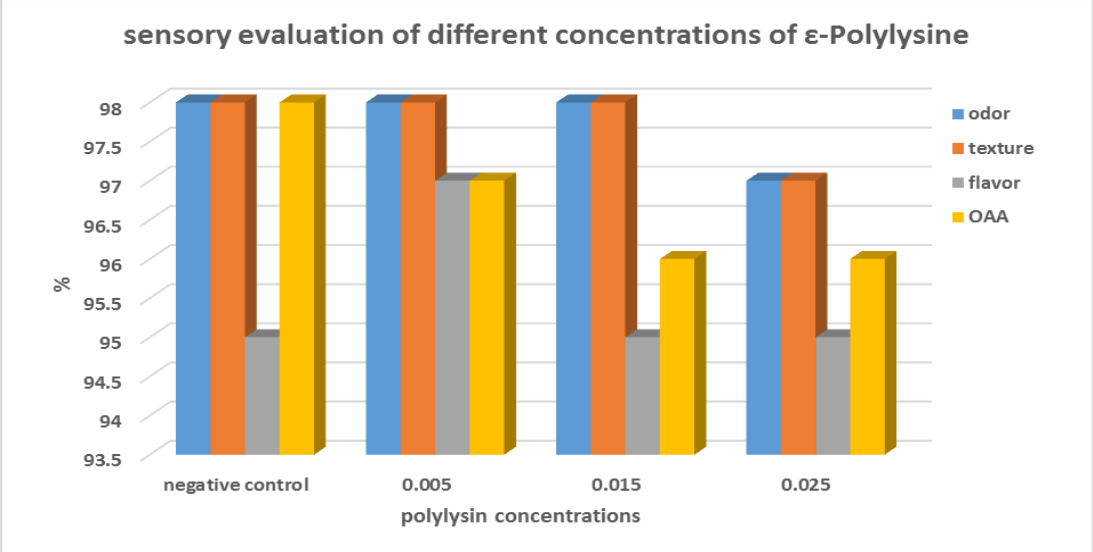


Fig. 3: The organoleptic impact of different concentrations of ε-Polylysine inoculated in yogurt.

DISCUSSION

The antibacterial cationic peptide known as ε-PL is a natural biological product, and a polymer containing a large number of free amino groups (Hosomi et al., 2015). It is an environmentally friendly and renewable resource, which has been used in the food and medical fields as a natural preservative and drug carrier (Zhu et al. 2010; Zhang et al. 2018). In this study, the active functional groups of ε-PL were detected by FTIR. As shown in Fig. 1, the peaks at 3442.96 and 2854.53 cm^{-1} were assigned to the absorption of N-H and C-H overlap, while those at 1746.60 and 1636.87 cm^{-1} were attributed to the stretching and scissoring vibration absorption of N-H groups, and to the stretching vibrations of amides I and II. The peak at 1465.55 cm^{-1} was assigned to the stretching vibration absorption of C=O, and peaks at 1377.86 and 1156.08 cm^{-1} corresponded to the absorptions of O-H and α,β unsaturation, respectively. These findings are in line with those reported in Hosomi et al. (2015) and Xu et al. (2021), and they revealed that the ε-PL used in this study has many active functional groups that could explain its antimicrobial activity and characterized for it.

According to the FDA (2010) ε-PL can be used as food additive at levels ranging from 0.005 to 0.06% (w/v) so, in this study, it was used at lower concentrations (0.005, 0.015, and 0.025%). The microbial species chosen in this study are considered major food-borne pathogens and they cause a large number of health-related concerns, including food-poisoning outbreaks, in Egypt. In order to solve these issues, it was chosen to specifically conduct the experiment on yogurt, which is considered the most popular food among immunocompromised consumers (children or elderly). Figure 2 shows that ε-PL has an antimicrobial activity against different types of bacteria, such as *E. coli* (Gram-negative), and *S. aureus* and *L.*

monocytogens (Gram-positive). The peptide could reduce the *E. coli* within the storage period, achieving a complete reduction four days after the yogurt was inoculated with ε-PL at a concentration of 0.025% at refrigeration temperature; whereas, at the same concentration, it could suppress *S. aureus* and *L. monocytogens* on the second day after inoculation. These results are in line with those presented in Gao et al. (2013) and Tan et al. (2018), who reported that ε-PL can inhibit the growth of *E.coli* bacteria; and those in Shi et al., (2016) and Zahi et al. (2017), who described the bactericidal effect of ε-PL on *S. aureus* and *L. monocytogens*. Because ε-PL can remove the lipopolysaccharides (LPSs) of Gram-negative bacteria, that enhances their access to the cytoplasmic membrane, since LPSs normally provides a continuum of negative charge that protects the cell against different cationic antimicrobial peptides (Schmidtchen and Malmsten, 2013; Bo et al., 2014). Due to the electrostatic nature of the interaction, and the differences in the ability of ε-PL to access the membrane, the peptide's effect can be competitively inhibited by cations in the surrounding liquid. Based on the results of the present study, it can be further speculated that an increase in pH would decrease the positive charge of ε-PL, thus reducing the electrostatic interaction with cells and determining a concomitant reduction in antimicrobial activity. ε-PL is an interesting peptide with antimicrobial properties against a broad spectrum of microorganisms (properties determined by its very general mechanism of action). In contrast, it has a low cytotoxic activity against eukaryotic cells, due to the intrinsic differences in the composition of the phospholipid head groups of eukaryotic and prokaryotic cell membranes (Li et al., 2018; Liu et al., 2018 and Zhou et al., 2018).

In addition to the antimicrobial activity of ε-PL, the assessment of the sensory quality characteristics of yogurt is important to obtain an indication of the consumers' acceptance of new products. Figure 3 shows that there is no significant difference between the yogurt at the highest ε-PL concentration (0.025%) and the control. Based on the panelists' evaluation, ε-PL concentrations of 0.005, 0.015, and 0.025% had 97, 96, and 96% OAA, respectively, which was almost in line with the OAA values obtained for the control yogurt without any additives (98%). This is due to the fact that the yogurt containing ε-PL was white, had a light sugary taste and an increased firmness of its texture, which made the product acceptable to consumers. Shih et al. (2006) mentioned that ε-PL could confer yogurt a good texture and firmness because it is characterized as a biodegradable fiber. Yogurt has the features of sweetness, astringency, harshness, in addition to a characteristic taste, preferred aroma, and mouth feel (Shih et al., 2006; Tamime and Robinson, 2007; Tribby, 2009 and Rajapaksha et al., 2013).

The results of this study indicate that yogurt samples with 0.025% (W/V) ε-PL presented a higher antibacterial activity compared to those at other concentrations, and had a good acceptability. The control yogurt ranked lower for bacterial growth and nearly with overall acceptability. In addition, it has been recently shown that the use of chemical preservative can be harmful, hence, there is a trend to use natural rather than chemical preservatives. The former are nontoxic, biodegradable, and biocompatible to humans. Furthermore, ε-PL provides many benefits to humans (FDA, 2010).

CONCLUSION

The present study detected many functional groups in ε-PL by FTIR, confirming its active properties against harmful bacteria. This peptide has a good antimicrobial activity against Gram-negative and Gram-positive bacteria, and a good OAA. Therefore, the addition of ε-PL to yogurt at a concentration of 0.025% is recommended to avoid the growth of most food-borne pathogens, to extend the shelf-life of dairy products, and obtain a better OAA.

Declarations

Competing interests:
The authors declare that they have no competing interests.

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Conflict of interest: none.
Ethics approval: This research did not involve experiments on humans or animals and received the ethical approval of the Animal Health Research Institute, Agriculture Research Center, Egypt.
Consent to participate: Not applicable
Consent for publication: Not applicable

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