Research Article

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The Co-Isolation of Lactic Acid Bacteria (LAB) and A Related Pathogenic Strain from *Pangasius Nasutus*

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ABSTRACT

Catfish species *Pangasius nasutus*, or locally known as *Patin Buah* is one of a popular aquaculture product in Malaysia. Probiotic strain is an attractive alternative to conventional antibiotics in managing aquaculture diseases. *Pangasius* can be a source of bacterial strain in developing suitable probiotic useful in *Pangasius* rearing. This study highlights on the isolation of Lactic Acid Bacteria (LAB) strains from the selected tissues from *P. nasutus* which are the heart, stomach, and intestines. These strains were subjected to morphological, biochemical, and genotypic characterisations. Three different strains were isolated from fish tissues, H-Hn from heart; S-Hn from stomach; and I-Sk from intestine. Biochemical characterisation were consistent with ribosomal rRNA sequencing, in which strain H-Hn (acc. No. MW504962) is highly similar (100%) to *Lactococcus lactis,* and both I-Sk (MW504964) and S-Hn (MW504963) are similar (99%) to *Lactococcus garvieae*. All strains appeared to be non-spore forming, non-motile, Gram-positive coccus, catalase and citrate negative, and fermenting sugar. Unlike H-Hn, both H-Sk and I-Sk strains were able to grow at 6.5% NaCl and at higher temperature (45°C). The antimicrobial properties were assessed using agar disk diffusion assay against several indicator organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*). These strains exhibited varying degree of inhibitory properties against the indicator organisms. Strain H-Hn inhibited *E. coli* and *V. parahaemolyticus*. Meanwhile, strains I-Sk and S-Hn showed a rather broad-spectrum inhibition. From *P. nasutus*, other *L. lactis* strain, a pathogenic species from *L garvieae* could also be found especially in their gut tissues. The presence of *L. garvieae* in commercial *P. nasutus* should raise some concern to those who like to consume this fish. Meanwhile, the LAB strains isolated has probiotics potential that can be commercially used in the managing fish diseases in aquaculture.

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Introduction

Pangasius nasutus is a ray-finned fishes belonging to shark catfish family *Pangasiidae* which can be found in freshwater habitat especially in South and Southeast Asia. In Malaysia, it is locally known as 'Patin Buah' and is popular when cooked with fermented durian called *tempoyak*, forming an important delicacy, especially in towns located along Pahang River. Increase in fish demand, traditional fish farming has moved

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into commercially intensive farming. Like many other fishes, *P. nasutus* was also subjected to captive breeding. These results in overcrowding and poor water quality which make them vulnerable to diseases outbreaks. While the aquaculture industry has also suffered losses due to viral and microbial diseases [1], the diminishing quality of rivers or estuaries might bring this fish to inevitable extinction [2].

Antibiotic and chemical drugs have been a method of choice in aquaculture disease management. However, the frequent reliance on chemicals and antibiotics has resulted in the emergence of antibiotic-resistant pathogens making antibiotics no longer effective [3]. In addition, poor management of aquaculture waste has given rise to public health concern [4]. As a substitute, probiotic supplementations have been suggested as an effective and environmentally safe methods to combat diseases. Probiotic is a life microorganism that positively affects the host organisms, usually belongs to Lactic acid bacteria (LAB) [5]. Probiotics which inhabit the fish or animal gut, offers many advantages include enhancing the immune system, feed efficiency, providing nutrition and improving the digestive system of the host. Moreover, antimicrobial substances called bacteriocin commonly produced by probiotic microorganisms could inhibit bacterial infections in fishes by restricting the growth of pathogens [6].

Host associated probiotics are microorganism collected from healthy host which can be used as an effective probiotics for the same host organism [7]. In this study, LAB strains were screened from internal organs of *Pangasius nasutus*; heart, stomach and intestines. With the isolated LAB strains, their phenotypic and genotypic characterisation were carried out. Finally, the antimicrobial activities of the isolated LAB strains against several indicator organisms were conducted to determine their potential antagonisms against pathogenic strains. The LAB strain showing inhibition against selected human or fish pathogen is worth of further characterisation for future aquaculture use. Even though strains from Gram-positive non-spore forming of typical *Lactococcus lactis* was presence, and there was a co-presence of a related pathogenic strain *L. garvieae* from a commercial and healthy *P. nasutus*.

Materials and Methods

Sample preparation

A fish sample (*P. nasutus*) approximately ~1.1 m length and ~1.18 kg) was purchased from a local fish market near Peramu, near Pekan, Pahang, Malaysia (see **Fig 1**). The

fish was first dissected, and its internal organs (heart, stomach and intestines) were extracted, weighed and rinsed with sterile distilled water. The organs were homogenized in phosphate buffered saline (PBS). The mixtures were vortexed and left in shaker at 30°C overnight. The homogenized samples were serially diluted with 0.85% NaCl, and the fifth dilution (10^{-5}) was spread on de Man, Rogosa and Sharpe (MRS) agar containing (in %w/v) 1.0% peptone, 1.0% beef extract, 0.4% yeast extract, 2.0% glucose, 0.5% sodium acetate trihydrate, 0.1% Tween 80, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate, 0.005% manganese sulfate tetrahydrate, and 1.0% agar (pH adjusted to 6.2 at 25 °C). The plates were left to incubate at 30°C for 48 h, the colonies formed were sub-cultured on MRS agar plates until single colonies; stock culture were prepared with 15 % (v/v) glycerol and stored at -80°C until further uses [8].

Fig 1 The fish *P. nasutus* sample used in this works

Phenotypic characterisation

For phenotypic characterisation, these isolates were subjected to Gram staining, catalase test, salt tolerance test, Simmon citrate test, temperature endurance test, and Triple Sugar Iron (TSI) test [9]. For the catalase test, a drop 30%(v/v) hydrogen peroxide was added to cell sample smeared onto a glass slide. The oxygen bubbles formation indicated a positive result. Simmons citrate test, Simmons citrate agar was used to test their capability to ferment citrate as their source of carbon [10]. TSI medium will test capability to ferment glucose, sucrose, and lactose and H_2S production. For salt tolerance test, Brain Heart Infusion (BHI) medium at 6.5% NaCl was used to select for

salt-tolerant microbes. Ability to grow at different temperatures was tested by inoculating the isolate into MRS broth and incubated at 10°C and 45°C for 72 h.

Antimicrobial activities

For antimicrobial activities, the disc diffusion method [11] was carried out with modification using the following indicator organisms: *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and fish pathogen *Vibrio parahaemolyticus*. All these strains were derived from internal collection, kept at Kulliyah of Science, International Islamic University Malaysia. The indicator organisms were sub-cultured until single colonies on Mueller-Hinton (MH) agar (1.5 g starch, 17.5 g casein hydrolysate, 300 g dehydrated infusion from beef and 17.0 g agar per liter of water, pH 7.3), then cultivated on MH broth at 25°C for 24h. Then, an inoculum (at 0.5 McFarland standard; ≈1.5 × 10^8 CFU/ml) were streaked onto MH agar surface and incubated at 25°C for 12-24 h. Sterile paper discs pre-impregnated in liquid broth of the isolated LAB (≈1.5 × 10^8 CFU/ml) were placed on the corresponding MH agar plates. The plates were incubated 37°C for 24 h before which the diameter inhibition zones formed surrounding the discs were measured from the edge of the paper disk. All tests were carried out in triplicates.

Genotypic characterisation

The genotypic identification for the isolates was carried out based on 16S rRNA sequencing [12]. The 1.5 gene fragment corresponds to 16S rRNA gene was amplified using universal primers, for Forward: 5'-AGAGTTTGATCCTGGCTCAG-3', and for Reverse: 5'-CCGTCAATTCCTTTGAGTTT-3' [13]. PCR Amplification (reaction volume of 50 µl) was carried out with DNA template (5 µl), each primer (5 µl, 10 pmol) and 5.0 µl of a 10x PCR master-mix buffer at 50 mM each dNTP (dATP, dCTP, dGTP and dTTP), 3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl, pH 8.3 and 0.1 U. μl^{-1} Taq polymerase. The amplification was set at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 55 °C for 40s, 72 °C for 1 min with a final extension at 72 °C for 5 min and holding at 4 °C for 1 h. After amplification, 5.0 µl PCR products was subjected to agarose gel electrophoresis (1.0% agarose, 1x TAE buffer, 100 V) for 2 h and the 1 kb molecular size standard ladder (Fermentas, Lithuania) was used. The gel was prestained with ethidium bromide (1.0 µg/ml) and analysed using gel imager (Alpha Imager). Each PCR

product was extracted, and the final sequencing was carried out using a local sequencing agency (Apical Scientific Laboratories Sdn. Bhd. Malaysia).

Sequence homology and analysis were performed using an on-line BLASTN tool program available at the National Centre for Biotechnology Information, NCBI, accessible at *http://www.ncbi.nlm.nih.gov*. The sequences were also deposited to NCBI database of which each was then provided with a corresponding accession number: H-Hn for MW504962; S-Hn for MW504963; and I-Sk for MW504964. To construct a phylogenetic tree, an online tool *NGphylogeny.fr* was used which is available at https://ngphylogeny.fr/ [14]. The 16S RNA sequences were aligned using Clustalw, and the phylogram was constructed using Maximum likelihood (approximate likelihood ratio test of SH-like), at bootstrapping of 100 and substitution rate of 4 (PhyML). The phylogram was viewed using interactive tree of life (iTOL v5).

Results and Discussion

Morphological and biochemical studies

The morphological and biochemical characterisation are shown in Table 1. They are all Gram positive, and all strains appeared coccus in shape (see Fig 2). All were recognized to be non-motile and catalase-negative for the lack of catalase activity. Simmons citrate agar test results suggested that all strains were citrate negative due to inability to use citrate as their carbon and energy source [15]. Moreover, all strains were also incapable of producing hydrogen sulphide (H_2S) that causes blackening. These strains belong to LAB as they are facultative anaerobes and do not require oxygen to grow. Lack of gas production indicated that these strains were homofermentative rather than heterofermentative [16]. Based on all these observations, these strains could belong to *Lactococcus sp.*

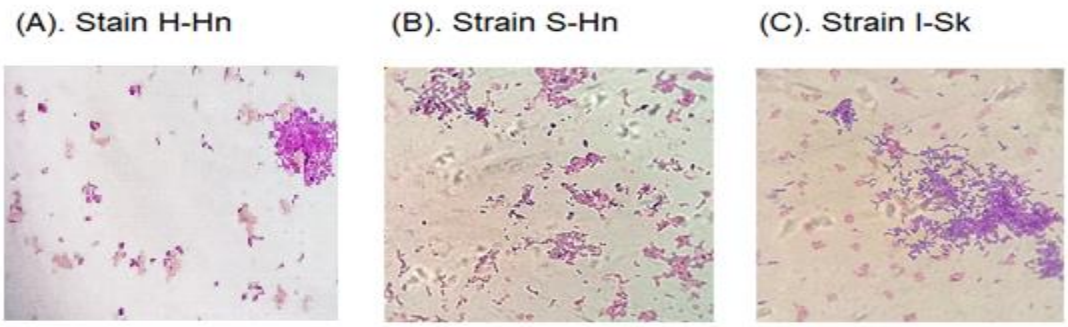

Fig 2 (Panel A-C) Morphology staining for the three LAB strains collected from *P. nasutus*; 100x, oil immersion (Nikon)

Temperature and salt tolerances

As for the salt tolerance and temperature endurance test, H-Hn strain could not tolerate 6.5% NaCl broth, confirming that it is salt intolerant and was not able to grow at either 10°C or 45°C. On the other hand, strain S-Hn and I-Sk were salt tolerant as they were able to thrive in 6.5% NaCl broth. For the temperature endurance test, strain S-Hn only grew at 45°C but not 10°C suggesting that it can tolerate higher, but not lower temperature. Meanwhile, strain I-Sk was able to tolerate both temperatures. Although, all strains could potentially belong to the *Lactococcus* genus, strain S-Hn and I-Sk are rather different from H-Hn. For instance, it was also reported that *L. lactis* strain isolated from Cuvier's beak whale could also grow at 7% salt and at 40°C. These observations were considered common for marine derived *L. lactis* [17], and recognized as the properties of so-called marine derived Lactic acid bacteria [18, 19]. Our subsequent 16S rRNA sequencing had confirmed they are not belonging to the same species.

Table 1 Phenotypic test results for three LAB strains

Test	H-Hn	S-Hn	I-Sk
Catalase test			
Gas bubbles	Negative	Negative	Negative
Simmon's Citrate test			
Medium Colour	Green	Green	No change
Triple Sugar Iron (TSI) test			
Slant colour/Reaction	Yellow/Acid	Yellow/Acid	Yellow/Acid
Butt colour/Reaction	Yellow/Acid	Yellow/Acid	Yellow/Acid
Gas bubbles	None	None	None
H_2S production	None	None	None
Motility	Non motile	Non motile	Non motile
Salt tolerance test			
6.5% NaCl	None	Yes	Yes
Temperature dependent test			
10°C	None	None	Yes
45°C	Negative	Positive	Positive

Antimicrobial properties

Based on result from disk diffusion as shown in Table 2, these strains demonstrated different level of inhibition zones against the indicator strains used (see also Fig. 3).

Strain H-Hn showed inhibition against *E. coli* and *V. parahaemolyticus*. On the other hand, strains S-Hn and I-Sk were able to show rather broad-spectrum inhibition against both Gram-positive and -negative strains. Strain S-Hn showed inhibition against almost all indicator strains, except for *P. aeruginosa.* Strain I-Sk showed quite similar profile except for *Salmonella typhimurium* and *P. aeruginosa.*

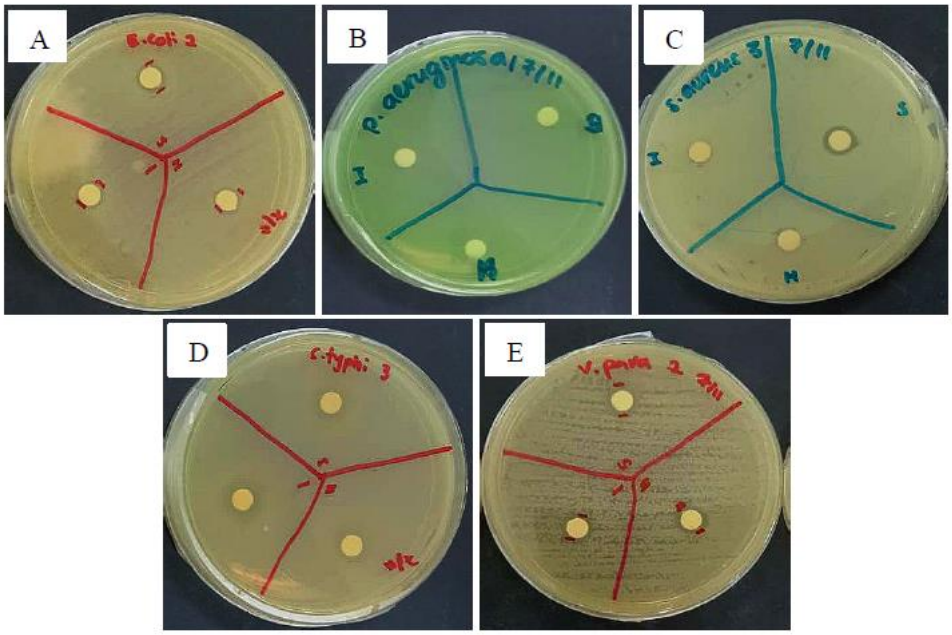

Fig 3 Antimicrobial activities based on disk diffusion method for three isolated LAB strains (H for Hn, S for S-Hn and I for I-Sk) on MH plates with different indicator organisms; (A) *Escherichia coli,* (B) *Pseudomonas aeruginosa,* (C) *Staphylococcus aureus,* (D) *Salmonella typhimurium, and* (E) *Vibrio parahaemolyticus*

The ability of S-Hn and I-Sk strains to inhibit both Gram-positive and negative bacteria indicated that it showed a broad-spectrum inhibition. Sensitivities of *P. aeruginosa* was however dependent on the type of assay being used [20]. Nevertheless, this observation is consistent with many observations that LAB inhibits Gram negative bacteria [21]. In one of our previous work, strain *L. lactis* strain isolated from black tip shark was able to produce a potent antimicrobial compound, bacteriocin [22]. With this antimicrobial action, for example, strain H-Hn has potential to be used in the Pangasius aquaculture. Furthermore, probiotic strains isolated from corresponding fish host are considered more efficient than those of from non-fish hosts, as the so-called ‘host-associated’ probiotics offers many benefits to the host organism [7, 23].

Presence of *Lactococcus garvieae* in *P. nasutus*

Within *Lactococcus* genus, there is a closely related pathogenic species belonging to *L. garvieae*. This is the first report on the presence of *L. garvieae* in *P. nasutus*. This species was reported to be found in other fishes and other aquatic species [24], and it was commonly associated with haemorrhagic sepsis outbreak in warm water fishes such as the rainbow trout [25]. Ability to grow at >4% salt and high temperatures (40°C) is consistent with other observations made elsewhere, however there were strains which cannot grow in these conditions [26]. The differences in temperature and salt tolerances between *Lactoccocus* strains is not clearly distinguishable. Nevertheless, the close presence of *L. lactis* and its pathogenic counterpart *L. garvieae* in fish internal organs is an intriguing fact, but this is not that new. The occurrence of haemorrhagic sepsis due to Lactococcosis in fish should be a reason for concern. Therefore, its presence in fish food should be an alert as Lactococcosis is an emerging disease responsible for major economic losses in warm water aquaculture [27, 28].

Table 2 Disk diffusion test results for the 3 LAB strains against indicator organisms

Indicator organisms	**Zone of inhibition (mm) ± 0.1mm**		
	H-Hn	**S-Hn**	**I-Sk**
Staphylococcus aureus	-	10.7	9.5
Salmonella typhimurium	-	12.0	-
Escherichia coli	7.3	9.2	8.0
Pseudomonas aeruginosa	-	-	-
Vibrio parahaemolyticus	7.5	11.0	9.3

Phylogenetic studies

Based on rRNA sequencing, the BLASTN hit search results as depicted in Table 3, strain H-Hn is similar to *L. lactis* (100%); meanwhile, strain S-Hn and I Sk are similar (>99%) to *L garvieae.* This also supported that S-Hn and I-Sk are slightly different from H-Hn we observed during phenotypic tests. A phylogenetic tree, as shown in Fig 4, was also constructed to visualize how the relationship between all three strains radiate among other *Lactococcus sp.* strains. Strain H-Hn is clustered in *L. lactis* group, meanwhile S-Hn and I-Sk are clustered in a group that contains *L. garvieae.* The presence of *L. lactis* and *L garvieae* in fish internal organ is intriguing, being the fact that these strains are of animal origin. This also could reveal the possible dietary source used by local farmers in rearing of *P. nasutus*. Due to costly fish meal, it is more

economical among local farmers to use chicken or broiler offal in fish feeding [29]. Thus, the consumption of commercially purchased *P. nasustus* can potentially lead to public health concern.

Table 3 Blast hit search for 16S RNA sequences of the three LAB strains

Strain	Top hits	Total score	Coverage (%)	E-value	Percentage identity	Accession number
H-Hn	*Lactococcus lactis*	2556	100	0.0	100.0	NR_040955.1
S-Hn	*Lactococcus garvieae*	2668	99	0.0	99.8	NR_113268.1
I-Sk	*Lactococcus garvieae*	2677	99	0.0	100.0	NR_113268.1

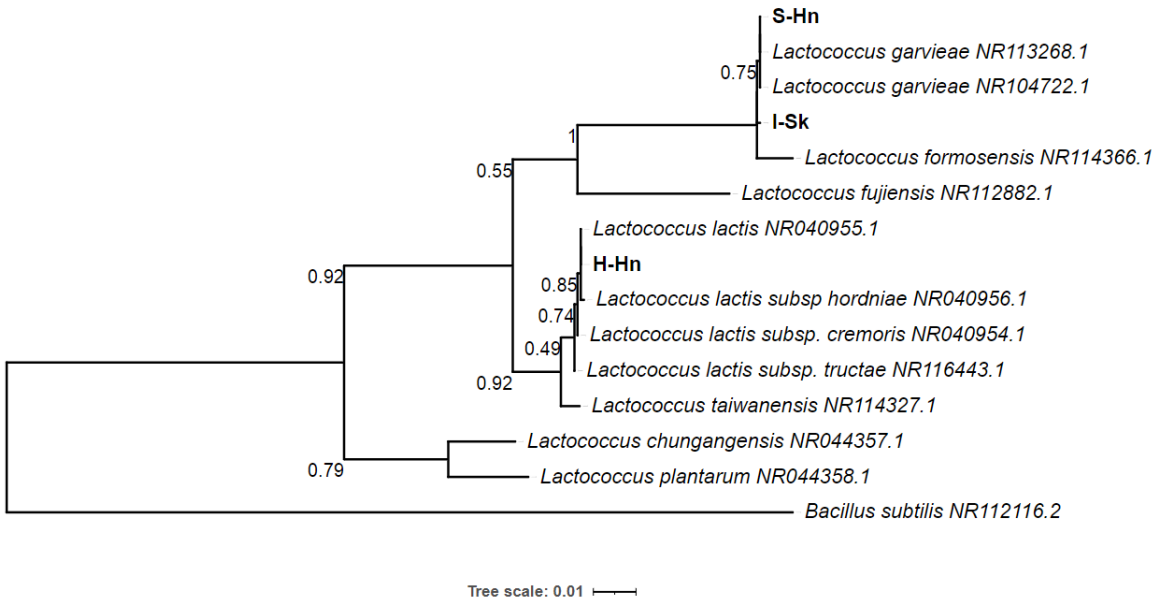

Fig 4 Phylogram for 16S rRNA genes sequences for the three LAB strains in relation to other Lactococcus sp. strains. This tree was constructed using Maximum likelihood at 100 bootstrapping and 4 substitution rates. The scale bar 0.01 is substitution number per nucleotide position, the number at each node is relative node height (base substitution per site). *Bacillus subtilis* represents as an Outgroup

Conclusions

Our work has isolated three LAB strains from different internal organs of *P. nasutus.* These strains are Gram positives coccus, catalase negative, non-motile and non-spore former. The phenotypic studies supported our genotypic studies, confirming the strain

H-Hn which was isolated from heart belongs to *L. lactis*. Meanwhile, both of strain S-Hn, isolated from stomach, and strain I-Sk, isolated from intestines, belong to *L. garvieae.* These strains showed different antagonisms against several indicator organisms. The presence of *L. lactis* and its pathogenic strain *L. garvieae* in the gastrointestinal organs of *P. nasutus* is an interesting phenomenon. The fish is likely to acquire these strains from dietary source, and thus, there is a safety implication in consuming this fish. Meanwhile, the isolation of *L. lactis* strain can potentially be used to develop host associated probiotic in *Pangasius* aquaculture.

Abbreviations
LAB: Lactic acid bacteria; CFU: Colony Forming Unit; *P. nasutus: Pangasius nasutus*; *L: Lactococcus*; MH: Mueller-Hinton; MRS: Man, Rogosa and Sharpe; rRNA: Ribosomal RNA; TSI; Triple Sugar Iron; BHI: Brain heart infusion; PCR: Polymerase Chain Reaction.

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Data Availability statement
The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards
Conflict of interest
The authors declare no conflict of interest.

Ethical standards
The study is proper with ethical standards.

Authors' contributions
In this work, the laboratory works were conducted by Nurul Sakinah and Nur Hannah. While Dr Abdul Hamid has supervised their works, Dr. Nur Nazifah as the co-supervisor assisted in sponsoring and overseeing the work on fish. The manuscript was edited and finalised by Dr Abdul Hamid.

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