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# *IN VITRO* ASSESSMENT OF PROBIOTIC POTENTIAL AND SAFETY OF TWO *Lactobacillus gasseri* STRAINS ISOLATED FROM MOTHER'S BREAST MILK AND INFANT'S FECES IN VIETNAM

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SUMMARY

Probiotics have multiple beneficial effects on human health, including modulation of the immune system, inhibition of enteric pathogens, and prevention of metabolic disorders. Lactic acid bacteria (LAB) are the most common bacterial strains used for probiotic production. Among those, species belonging to the *Lactobacillus acidophilus* complex, especially *L. gasseri,* were well characterized and reported for probiotics. This study evaluated the potential probiotic characteristics of two *L. gasseri* strains (designed as VTCC 12791 and VTCC 12792) isolated from a pair of mother's breast milk and infant's feces. Both strains completely survived in the simulated gastric juice, highly tolerated the simulated intestinal juice (81-84%), strongly adhered to the HT-29 cell (23.5% for VTCC 12791 strain and 5.5% for VTCC 12792 strain), and had antagonistic activity against food-borne pathogens of *Aeromonas dhakensis, Vibrio vulnificus,* and *Listeria monocytogenes*, which demonstrated better beneficial properties than those of the commercial reference *Lacticaseibacillus casei* Shirota (LcS) strain. Additionally, both strains exhibited xanthine oxidase inhibitory activity (approximately 24%), which can lead to reducing serum uric acid. Regarding the safety aspect, the strains were sensitive to several antibiotics of ampicillin, vancomycin, tetracycline, chloramphenicol, erythromycin, and possessed alpha-hemolytic activity, which were similar to those of LcS strain. Our results suggested that *L. gasseri* VTCC 12791 and VTCC 12792 are potential candidates for producing probiotics and functional foods. Further *in vivo* studies are needed to prove their potential health benefits, especially in the treatment of hyperuricemia.

**Keywords:** *Infant's feces, mother's breast milk, lactic acid bacteria, Lactobacillus gasseri, probiotics, xanthine oxidase inhibitor*

## INTRODUCTION

*Lactobacillus gasseri* is a species of lactic acid bacteria (LAB) that belongs to the genus *Lactobacillus*, a group of Gram-positive, catalase-negative, non-spore-forming, and rod-shaped bacteria. *L. gasseri* is commonly found in the lower genital tract of human females but can also be isolated from other human body sites, such as the

mouth and intestinal tract or the intestines of animals (Zheng *et al.*, 2020). Currently, *L. gasseri* is classified into the *L. acidophilus* complex, which has been widely used to develop probiotic products (Selle, Klaenhammer, 2013).

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001), and the functional benefits of probiotics are numerous and have been widely studied. Probiotics have been shown to modulate the composition of gut microbiota, which can create a strong barrier for the body against harmful bacteria and reduce the risk of enteric infection. Moreover, probiotics can improve metabolic function, such as reducing inflammation and lowering cholesterol levels, improving mental health (Hill *et al.*, 2014). Furthermore, probiotics have a potential application in ameliorating hyperuricemia, which is a major risk factor for the progression of gout, cardiovascular disease, diabetes, chronic kidney disease, and non-alcoholic fatty liver.

The widespread colonization of *L. gasseri* to the human mucosa, including the gastrointestinal tract (GIT), lower female urogenital tract, and breast milk, indicates its prevalence as a commensal in healthy individuals and may contribute to probiotic activity (Selle, Klaenhammer, 2013). Some LAB strains, including *L. gasseri,* have been reported that they possess uric-acid lowering capability by producing xanthine oxidase inhibitory (Chen *et al.*, 2017) or assimilating purine nucleosides (Li *et al.*, 2014; Wang *et al.*, 2019). The effectiveness of probiotic bacterial strains, such as *L. plantarum* GKM3, *L. brevis* DM9218, and *L. gasseri* PA-3, in lowering serum uric acid levels has been evidenced through animal studies and clinical data (Ni *et al.*, 2021).

Several commercial products of probiotic *L. gasseri* are currently available in the market. One of the best-known commercial strains is *L. gasseri* BNR17. This strain was isolated from human breast milk and has the ability to reduce blood glucose and cholesterol, which can be used in weight management and relieve irritable bowel syndrome (IBS) symptoms (Jung *et al.*, 2013; Kim *et al.*, 2018). Additionally, Ecovag product containing *L. gasseri* EB01 has been used for the prevention and treatment of vaginal dysbiosis (Marcotte *et al.*, 2017).

The gut microbiota composition attributed to various lifestyles and environmental factors can vary in different geographical regions. Therefore, it is necessary to find a suitable probiotic candidate from the indigenous population. Currently, many probiotic products are available on the Vietnamese market, but most of these products originate from foreign countries. During our pilot investigation on the diversity of LAB in the mother's breast milk and infant's feces, we isolated and identified two *L. gasseri* strains from a pair of mother and infant. In this study, we aimed to evaluate the probiotic characteristics of these strains.

## MATERIALS AND METHODS

### Bacterial strains and cell line

According to the protocol approved by the Ethical Committee in Biomedical Research at the University of Medicine and Pharmacy, Vietnam National University, Hanoi (No. 23/2023/CN-HDDD), two strains of *L. gasseri* were isolated from the

breast milk of a 31-year-old mother and her infant feces living in a suburban of Hanoi. These strains were designed as VTCC 12791 (from breast milk) and VTCC 12792 (from infant feces) and deposited at the Vietnam Type Culture Collection (VTCC), Institute of Microbiology and Biotechnology (IMBT), Vietnam National University (VNU), Hanoi, Vietnam.

A reference strain of *Lacticaseibacillus casei* Shirota (LcS) isolated from a commercial fermented milk product (Yakult, Binh Duong, Vietnam) was used to compare the probiotic potential in all experiments.

The indicator pathogenic bacteria of *Aeromonas dhakensis* VTCC 70106*, Vibrio vulnificus* VTCC 70092, *Staphylococcus aureus* VTCC 12275, *Escherichia coli* VTCC 12272, *Salmonella enterica* VTCC 70080, *Listeria monocytogenes* VTCC 70147, and *Campylobacter jejuni* VTCC 70176 obtained from VTCC were used in antimicrobial activity testing.

A human colon adenocarcinoma cell line (HT-29) was provided by the Key Laboratory of Enzyme and Protein Technology (KLEP), Hanoi University of Science (HUS), Vietnam National University (VNU), Hanoi, Vietnam.

**Low pH tolerance**

A minor modified method described by Nami *et al.*, 2019 was used to evaluate the acid tolerance. After overnight cultured in MRS broth, bacterial cells were resuspended in PBS to obtain an optical density at 600 nm ($OD_{600}$) of 0.5. Then, 100 µL of cell suspension was mixed with 900 µL PBS (pH 2.5) and incubated at 37°C for 2 h. The initial and experiment aliquots were diluted and plated on MRS plates to check the viable colonies. The experiment calculation formula was as follows:

$$Survival\ rate\ (\%) = \frac{Final\ (\log CFU/ml)}{Initial\ (\log CFU/ml)}\ x\ 100$$

**Survival in simulated upper GIT**

The survival ability of strains in GIT was performed following the method described by Anh *et al.*, 2023 with minor modifications. The cells suspended in simulated gastric (pH 2.0) and intestinal juices (pH 8.0) were incubated at 37°C for 2 h and 6 h, respectively. The bacterial cells were counted before and after the treatment steps, and the survival rates of the testing strains were calculated as described above.

**Cell adhesion ability**

The intestinal adhesion ability of testing strains was evaluated following the method described by Anh *et al.* (2023).

**Auto-aggregation ability**

Auto-aggregation ability was measured according to the method described by Jena *et al.*, 2013. Bacterial suspensions were adjusted to $OD_{600}$ of 0.5 to standardize the bacterial concentrations. The bacterial suspension was incubated at room temperature for different periods (4, 6, and 24 h). The auto-aggregation percentage was determined using the following formula:

$$Auto-aggregation\ (\%) = \left(1 - \frac{A_t}{A_0}\right) \times\ 100$$

$A_t$: the absorbance at different times (t = 4, 6, and 24 h);
$A_0$: the absorbance at an initial time.

**Antimicrobial activity**

The antimicrobial activity of two isolates was determined following the method described by Anh *et al.* (2023).

**Xanthine oxidase inhibitory (XOI) activity**

The XOI activity of *L. gasseri* cultures was determined by quantifying the amount of uric acid formation from xanthine in the reaction mixture. For the sample test, 200 µL of reaction mixture consisting of bacterial CFS, Tris-HCl buffer (0.1 M, pH 7.5), and xanthine oxidase (60 µU/mL) were incubated for 10 min at 37°C. After adding oxonic acid potassium (1 mM), the reaction was added with xanthine (10 mM) as a substrate and subsequently incubated at 37°C for 30 min. The same amount of MRS medium and allopurinol (0.4 mg/mL) was used instead of bacterial CFS for negative and positive controls, respectively. Then, the reaction was stopped by adding 100 µL of 1N HCl before quantifying the uric acid concentration by high-performance liquid chromatography (HPLC). The reaction mixture was filtered through a 0.22-µm membrane, and injected into an Agilent 1260 HPLC system installed with an Agilent column Zorbax SB-aq (4.6 mm × 150 mm, 5 µm) and UV detection at 284 nm. The column temperature was constant at 30°C, and the mobile phase consisting of 0.02 M $KH_2PO_4$ was eluted at a flow rate of 1 mL/min (Wu *et al.*, 2021). Based on the obtained peak at the appropriate retention time, the uric acid concentration was calculated by extrapolating from a standard curve generated from uric acid concentration ranging from 0 to 400 µg/L. The percentage inhibition of XO was calculated by the following formula:

$$\%\ inhibition = \left(1 - \frac{A}{B}\right) x\ 100$$

A is the concentration of uric acid in assay with the *L. gasseri* sample;

B is the concentration of uric acid in assay with MRS medium.

**Antibiotic susceptibility**

Antibiotic susceptibility was carried out using the disc diffusion method and followed by Anh *et al.*, 2023. Nine antibiotics, including ampicillin (AMP, 10 µg), vancomycin (VAN, 30 µg), gentamicin (GMN, 10 µg), tetracycline (TET, 30 µg), chloramphenicol (CHL, 30 µg), erythromycin (ERY, 15 µg), clindamycin (CMN, 2 µg), ciprofloxacin (CIP, 5 µg), and streptomycin (SMN, 10 µg) were used on this test. The susceptibility of strains was classified according to the cut-off values recommended by (Charteris *et al.*, 1998).

**Hemolytic activity**

The hemolytic activity of two strains was tested by streaking the bacteria on the Columbia agar supplemented with 5% sheep blood. After incubation at 37ºC for 24 and 48 h, the hemolytic activity was described by observing the clear zone surrounding the colonies (β-hemolytic), a partial and green discoloration surrounding the colonies (α-hemolysis), or no zone around the colonies (γ-hemolytic) (Nami *et al.*, 2019).

**Statistical analysis**

All data was obtained from three independent experiments, with each

performed in duplicate. The results were expressed as mean ± standard deviation (SD). In addition, statistical ANOVA analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc.)

## RESULTS AND DISCUSSION

### Probiotic characteristics of two *L. gasseri* strains

During our pilot investigation, two lactic acid strains designated as VTCC 12791 and VTCC 12792 (https://vtcc.imbt.vnu.edu.vn/) were isolated from a pair of mother's breast milk and infant's feces, respectively. The 16S rDNA sequence of the strains was analyzed and deposited at the Genbank under accession numbers OP889360 (VTCC 12791) and OP889361 (VTCC 12792). Both 16S rDNA sequences were identical, and the bacterial strains were identified as *L. gasseri*, with 99.93 % identity to that of the type strain ATCC 33323.

Two strains *L. gasseri* were resistant to acid, and both strains retained about 100% viability after treatment at pH 2.5 for 2 h. At the same treatment condition, the reference strain LcS had a significant reduction of 4.47 log CFU/mL.

The viable cell numbers and survival rates of *L. gasseri* under the simulated GIT conditions are shown in Table 1. In the human stomach, the probiotic bacteria have to survive under not only the acidic condition but also the effect of the proteolytic activity of pepsin (Zhu *et al.*, 2006). Our strains showed excellent survivability with no reduction compared to the commercial strain LcS, which had a total loss of viability under artificial gastric conditions. Despite their high acid tolerance, our strains had a slight reduction (approximately a log of CFU/mL) in the pancreatic conditions, whereas the reference strain LcS showed excellent viability. Our study is in agreement with other reports that showed *L. gasseri* strains were generally well-tolerated to the human gastrointestinal tract (Martin *et al.*, 2005; Oh *et al.*, 2018; Rodrigues da Cunha *et al.*, 2012).

**Table 1.** The survival rate of two *L. gasseri* strains under GIT conditions. The average values and ± SD were calculated from three dependent experiments. Samples with different letters (compared within the same parameter) are significantly different at $p < 0.05$.

| Strains | Survival in gastric juice | | | Survival in intestinal juice | | |
|---|---|---|---|---|---|---|
| | Initial count (log CFU/mL) | 2 h (log CFU/mL) | Survival rate (%) | Initial count (log CFU/mL) | 6 h (log CFU/mL) | Survival rate (%) |
| VTCC 12791 | 6.80 ± 0.14 | 7.21 ± 0.28 | 105 ± 1.96$^{a}$ | 7.09 ± 0.26 | 5.96 ± 0.06 | 84 ± 2.81$^{a}$ |
| VTCC 12792 | 6.27 ± 0.02 | 7.01 ± 0.01 | 111 ± 0.16$^{a}$ | 6.75 ± 0.30 | 5.47 ± 0.17 | 81 ± 2.27$^{a}$ |
| LcS | 7.38 ± 0.03 | n.d | 0$^{b}$ | 7.30 ± 0.09 | 7.37 ± 0.05 | 100 ± 0.67$^{b}$ |

n.d: not detected.

To identify a potential probiotic candidate, it is important that the bacteria can colonize the human gut over a long period. Thus, the adhesion ability was performed on the HT-29 cell line. Both *L. gasseri* strains were strongly adhesive to the HT-29 cells. Strain VTCC 12791 showed a higher adhesion ability (23.5%) than strain VTCC 12792 (5.5%). This ability was much higher than commercial strain LcS **(**Fig. 1A). Our results are in agreement with previous studies, which showed strain *L. gasseri* LaI had a high adhesion capacity to both Caco-2 and HT-29 cell lines and had a function in protecting against enteropathogenic bacteria (Zawistowska-Rojek *et al.*, 2022). Strain *L. gasseri* JM1, isolated from a fecal sample of a breastfed healthy infant, also had an excellent capacity for adhesion to the Caco-2 cell line (Sun *et al.*, 2020).

Auto-aggregation is an essential characteristic of probiotic bacteria, enabling their adhesion to epithelial cells and mucosal surfaces, thereby facilitating their colonization in the gastrointestinal tract (Klopper *et al.*, 2018). Moreover, auto-aggregation also allows the bacteria to create a barrier and prevents pathogenic bacteria's adhesion (Tuo *et al.*, 2013). This property depends significantly on the incubation time. In our study, two *L. gasseri* strains exhibited a high degree of auto-aggregation at the time of 4 h (from 23.88% to 33.93%) and continuously increased over the study time. The highest percentage of auto-aggregation (approximately 95%) was observed after 24 h (Fig. 1B). There were significant differences in the auto-aggregation ability between our two tested strains and the control strain LcS at three different time points ($p < 0.0001$).

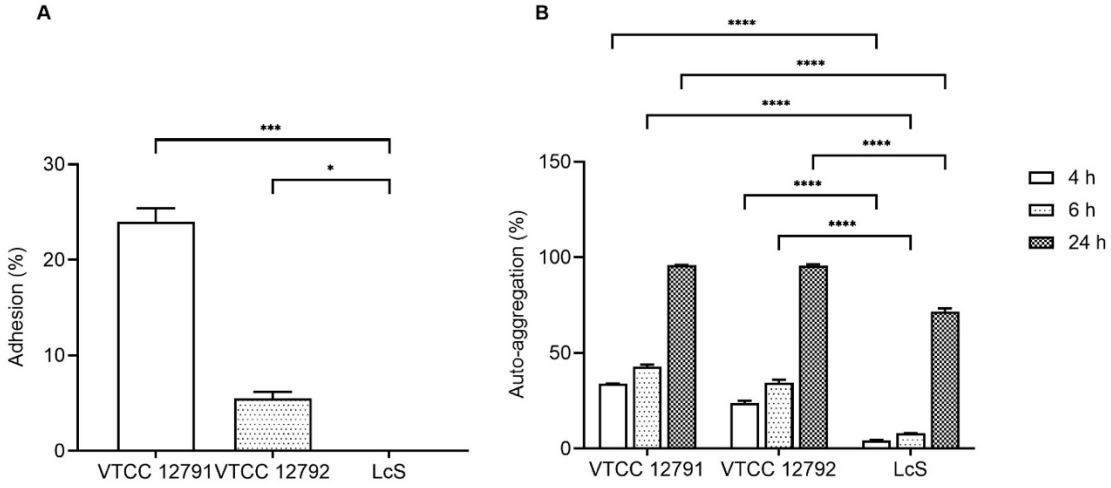


**Figure 1.** Adhesion ability (A) and auto-aggregation (B) of *L. gasseri* strains.

Similar results have been reported by other groups. Zawistowska-Rojek et al. (2022) reported the auto-aggregation of twelve LAB strains after 2 h incubation ranging from 8.4% to 21.4%, where the second highest value has been reached by *L. gasseri* LaI with 18.4% (Zawistowska-Rojek *et al.*, 2022). D'Alessandro et al. (2021) also reported that after 5 h of incubation, some *L. gasseri* and *L. crispatus* strains exhibited an

auto-aggregation value above 90%, which was significantly higher than the commercial control strain *L. rhamnosus* LGG, which only achieved an auto-aggregation value of 23% under the same conditions (D'Alessandro *et al.*, 2021).

One of the essential properties of probiotic candidates is its antagonistic activity against pathogenic microorganisms. *Lactobacillus* species have the ability to produce various antimicrobial compounds, such as organic acids, hydrogen peroxide, and bacteriocins, which have the potential to hinder the proliferation and establishment of pathogen bacteria in the gastrointestinal tract (Syngai *et al.*, 2016). Six human pathogens belonging to both Gram-positive and Gram-negative were employed in this study. Both *L. gasseri* strains exhibited inhibition activity against food-borne bacteria of *A. dhakensis, V. vulnificus,* and *L. monocytogenes*. No inhibitory activities were observed against Gram-negative *E. coli, S. enterica,* and *C. jejuni* (Table 2). The antimicrobial activity was not detected after neutralizing the CFSs (data not shown).

**Table2.** Antimicrobial activity of *L. gasseri* strains.

| Pathogens | Strains | | |
|---|---|---|---|
| | VTCC 12791 | VTCC 12792 | LcS |
| *L. monocytogenes* | + | + | + |
| *S. aureus* | - | - | - |
| *V. vulnificus* | + | + | + |
| *A. dhakensis* | + | + | + |
| *E. coli* | - | - | - |
| *S. enterica* | - | - | - |
| *C. jejuni* | - | - | - |

+ positive; - negative

*L. gasseri* CECT5714 and *L. gasseri* CECT5715 isolated from human breast milk are able to produce a high amount of lactic acid, which can inhibit the growth of pathogen bacteria, including *Salmonella choleraesuis*, *E. coli, S. aureus, L. monocytogenes*, and *Clostridium tyrobutyricus* (Olivares *et al.*, 2006). Rastogi et al. (2020) also reported the antagonistic property of three *L. gasseri* strains isolated from human milk and breast-fed infant feces. Although three strains have a strong inhibition against most of the food-borne pathogens such as *Shigella flexneri, Salmonella typhimurium, Enterococcus faecalis,* and *Shigella sonnei*, the antimicrobial activity reduced when the culture supernatant adjusted to pH 6.5 (Rastogi *et al.*, 2020). Our study had the same result, indicating that the production of acid lactic can have strong inhibitory activity against pathogenic bacteria.

## Safety assessments

One of the safety assessments for considering probiotics is antibiotic susceptibility. Probiotics should not carry

antibiotic resistance genes or transferable resistance determinants to other bacteria. However, some *Lactobacillus* strains are naturally resistant to certain antibiotics, while others may acquire resistance through horizontal gene transfer or other mechanisms. It is necessary to determine whether the resistance is natural or from other factors (Martin *et al.*, 2005).

In this study, both *L. gasseri* strains demonstrated susceptibility to ampicillin, vancomycin, tetracycline, chloramphenicol, and erythromycin. Resistance was observed against gentamicin, clindamycin, and ciprofloxacin. The LcS strains were sensitive to almost all antibiotics except streptomycin and vancomycin. Variable antibiotic susceptibility is presented in Table 3. In this study, the observed patterns of antibiotic resistance/sensitivity are in accordance with previous observations (Martin *et al.*, 2005; Rodrigues da Cunha *et al.*, 2012). The antibiotic resistance profile of the strain from the mother's breast milk matched with that of the infant strain. Compared to another study, matching isolates in 2 mother-infant pairs exhibited different antibiotic resistance profiles (Kozak *et al.*, 2015). Additionally, both *L. gasseri* strains showed alpha-hemolytic activity, similar to strain LcS (Data not shown). This activity might be due to lactic acid production (Chokesajjawatee *et al.*, 2020).

**Table 2.** Antibiotic resistance profiles of two *L. gasseri* strains.

| Antibiotics | Strains | | |
|---|---|---|---|
| | VTCC 12791 | VTCC 12792 | LcS |
| **Ampicillin (10 µg)** | S | S | S |
| **Vancomycin (30 µg)** | S | S | R |
| **Gentamicin (10 µg)** | R | R | S |
| **Tetracycline (30 µg)** | S | S | S |
| **Chloramphenicol (30 µg)** | S | S | S |
| **Erythromycin (15 µg)** | S | S | S |
| **Clindamycin (2 µg)** | R | R | S |
| **Ciprofloxacin (5 µg)** | R | R | MS |
| **Streptomycin (10 µg)** | MS | MS | R |

R: resistant; MS: moderately sensitive; S: sensitive

The breakpoints for the antibiotic sensitive/resistant in mm zone of inhibition: AMP: ≥16/≤12; CHL and ERY: ≥18/≤13; CMN: ≥12/≤8; TET: ≥19/≤14; GMN: ≥13/≤12; VAN: ≥17/≤14; CIP: ≥19/≤13; SMN: ≥15/≤11.

## Xanthine oxidase inhibitory activity

Hyperuricemia (HUA) is a metabolic disease, which is characterized by an abnormally high blood uric acid (UA) concentration (Yanai *et al.*, 2021). HUA is a major risk factor for the progression of gout, cardiovascular disease, diabetes, chronic kidney disease, non-alcoholic fatty liver, etc. (Agnoletti *et al.*, 2021). Urate-lowering pharmacological agents such as xanthine oxidase inhibitors (e.g., allopurinol) and

uricosuric agents (e.g., benzbromarone) are often used to treat hyperuricemia. However, long-term use will cause severe adverse reactions in the human body (Dalbeth *et al.*, 2019). Thus, it is necessary to explore novel products from natural sources that can be easily produced and safely administered.

In this study, both tested strains showed an XOI activity (ranging from 23.55 ± 0.64 to 24.30 ± 0.99%). The reference LcS strain did not show any XOI activity. These results are in agreement with Chen et al. (2017) that *L. rhamnosus* I21, isolated from the feces of a healthy infant, also had an XOI activity of about 26.74% (Chen *et al.*, 2017). Additionally, *L. gasseri* PA-3, which has been used in Japanese drinking yogurt, had an effective treatment for hyperuricemia in gout patients (Yamanaka *et al.*, 2019). Kano et al. (2018) demonstrated the mechanism for reducing serum uric acid levels caused by an assimilation of purine nucleosides, a precursor of uric acid (Kano *et al.*, 2018). Our study reported another mechanism in uric-acid lowering capability by producing XOI, which inhibits xanthine oxidase, an important enzyme in purine catabolism (Maiuolo *et al.*, 2016). This is the first report on the XOI activity of lactic acid bacteria isolated from Vietnam.

## CONCLUSION

Our findings revealed that both *L. gasseri* strains isolated from mother's breast milk and infant's feces possess desirable probiotic properties and safety. These strains are good candidates for further investigation *in vivo* studies to prove their potential health benefits, especially in the treatment of hyperuricemia.

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