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ANTAGONISTIC ACTIVITY OF *LACTOBACILLUS* AND *BIFIDOBACTERIUM* ISOLATED FROM YOGURT AGAINST PATHOGENIC ENTERIC BACTERIA

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Abstract
The consumption of yogurt, containing probiotic microorganisms like *Lactobacillus* and *Bifidobacterium*, has been associated with numerous health benefits, including improved gastrointestinal health. In this study, we investigated the antagonistic activity of Lactobacillus and Bifidobacterium strains isolated from commercially available yogurt against pathogenic enteric bacteria. This study investigated 50 samples of traditional Pakistani yogurt obtained from various regions of Peshawar, Khyber Pakhtunkhwa. Using MRS agar and standard microbiological techniques, *Lactobacillus* and *Bifidobacterium* strains were identified based on 16S rRNA sequencing. Antimicrobial activity was assessed using the well-diffusion method, with Gram staining confirming the Gram-positive nature of the isolates. Notably, *Staphylococcus aureus* exhibited positive tube coagulase, *Enterococcus* was catalase-positive, and *Salmonella typhi* tested positive in indole tests. *Lactobacillus* species were identified through PCR targeting the 16S rRNA gene. *Bifidobacterium* strains displayed significant activity against *S. aureus* (17mm inhibition zone) and moderate activity against *Escherichia coli*. Both *Lactobacillus* and *Bifidobacterium* demonstrated substantial antibacterial effects against *Salmonella typhi*, with lesser activity against *Enterococcus*. At 100 µg/ml concentration, both strains displayed potent antibacterial activity against S. aureus, followed by *Salmonella typhi* and *Enterococcus*, with moderate activity towards *E. coli*. Furthermore, phylogenetic analysis of the 16S rRNA gene sequences provided insights into the evolutionary relationships among the isolated strains. These findings underscore the potential of *Lactobacillus* and *Bifidobacterium* strains from traditional Pakistani yogurt as natural agents with antibacterial

properties, suggesting their potential in promoting gastrointestinal health and combating enteric infections.

Key words: Probiotics, Antagonistic activity, pathogen, Lactobacillus and Bifidobacterium

1. Introduction

In recent years, the exploration of probiotics, particularly those originating from fermented dairy products like yogurt, has captured significant scientific interest (Hesari, Darsanaki, & Salehzadeh, 2017; Servin, 2004). Probiotics, live microorganisms that confer health benefits when consumed in adequate quantities, have emerged as potential allies in promoting gut health. Among the vast array of probiotic candidates, *Lactobacillus* and *Bifidobacterium* species have garnered particular attention due to their ability to thrive in the gastrointestinal tract and exert beneficial effects (Servin, 2004). The human gut microbiota, a diverse community of microorganisms residing in the gastrointestinal tract, plays a pivotal role in maintaining overall health (El Kholy, EL SHINAWY, Meshref, & Korny, 2014). Disruption of the delicate balance of this microbial ecosystem, termed dysbiosis, has been linked to various gastrointestinal disorders and systemic conditions, underscoring the importance of preserving gut microbiota homeostasis (Delcaru et al., 2016). Probiotics, by modulating the composition and function of the gut microbiota, offer a promising avenue for restoring microbial balance and promoting gastrointestinal health (Delcaru et al., 2016; Karami et al., 2017). *Lactobacillus* and *Bifidobacterium* species, commonly found in yogurt and other fermented dairy products, are regarded as beneficial probiotics. These bacteria possess unique characteristics that enable them to survive the harsh acidic conditions of the stomach and colonize the intestine, where they can exert their beneficial effects (Karimi, Rashidian, Birjandi, & Mahmoodnia, 2018). Numerous studies have highlighted the potential health benefits of *Lactobacillus* and *Bifidobacterium* strains, including immunomodulation, enhancement of intestinal barrier function, and antagonism against pathogenic bacteria (Fijan, Šulc, & Steyer, 2018). Pathogenic enteric bacteria represent a significant threat to human health, causing a wide spectrum of gastrointestinal infections ranging from mild gastroenteritis to severe diarrheal diseases. The emergence of antibiotic-resistant strains further complicates the management of these infections, necessitating the exploration of alternative therapeutic approaches (Vélez et al., 2007). Probiotics offer a promising strategy for combating pathogenic bacteria through various mechanisms, including competitive exclusion, production of antimicrobial compounds, and modulation of host immune responses (Gharib, 2020). Given the widespread consumption of yogurt and other probiotic-containing foods, understanding the antagonistic activity of *Lactobacillus* and *Bifidobacterium* strains against pathogenic enteric bacteria is of paramount importance. Investigating the mechanisms by which probiotic bacteria inhibit the growth and virulence of pathogens can provide valuable insights into their potential therapeutic applications and help optimize their use in promoting gastrointestinal health (Gad, Abd El-Baky, Ahmed, & Gad, 2016; Gharib, 2020). Furthermore, elucidating the specific strains and mechanisms involved in the antagonistic activity of probiotics against pathogenic bacteria can inform the development of targeted probiotic formulations for therapeutic use (Al-Madboly & Abdullah, 2015). Additionally, understanding how probiotics interact with the host immune system and gut microbiota to confer protective effects against pathogens can provide insights into the broader implications of probiotic therapy for human health (Al-Madboly & Abdullah, 2015; Hossain et al., 2018). This study seeks to evaluate the antagonistic activity of *Lactobacillus* and *Bifidobacterium* strains isolated from yogurt against pathogenic enteric bacteria. By elucidating the mechanisms underlying probiotic-mediated inhibition of pathogen growth, this research aims to contribute to our understanding of the role of probiotics in maintaining gut health and preventing gastrointestinal infections.

2. Materials and Method

2.1. Samples Collection

Samples were collected from both locally sourced and commercially available yogurt varieties (randomly chosen from cow and buffalo milk). The sampling method employed was based on random convenience. Criteria for selection included maintaining samples at 4ºC and transporting them to the laboratory within 24 hours. Bacterial isolates were derived from screening 50 traditional Pakistani yogurt samples, acquired from various locations and villages across Peshawar, Khyber Pakhtunkhwa, encompassing urban and rural areas. The yogurt samples originated from diverse sources, including both cow and buffalo milk.

2.2. Isolation and Identification of Lactobacillus and Bifidobacterium Species

De Man Rogosa Sharpe (MRS) agar was used to culture lactic acid bacteria from yogurt samples, which were collected aseptically and transported to the lab. Colonies were isolated and sub-cultured on MRS agar plates. Identification involved Gram staining for cell morphology and biochemical tests. Cultured isolates go through Gram staining to determine their Gram status. Biochemical tests, including the oxidase, catalase, and indole tests, were conducted for further identification. Positive reactions indicated the presence of specific enzymes characteristic of *Lactobacillus* and *Bifidobacterium* species.

2.3. Identification of Isolated Bacteria by Molecular Methods

The identification of *Lactobacillus* and *Bifidobacterium* strains was confirmed by analyzing the 16S rRNA sequences. Bacterial DNA was isolated from all samples post biochemical test confirmation. PCR was conducted using specific primer sequences: 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-CCGTCAATTCCTTTGAGTTT-3' (reverse) for *Lactobacillus*, and 5'-CTCCTGGAAACGGGTGG-3' (forward) and 5'-GGTGTTCTTCCCGATATCTACA-3' (reverse) for *Bifidobacterium*. PCR was carried out in a 25 µl final volume, with 35 cycles of amplification. Electrophoresis of PCR products on a 1.5% agarose gel followed by ethidium bromide staining enabled visualization. The 900 bp amplified fragments were sequenced by Macrogen Company (Korea) using the ABI PRISM 7700 Sequence Detection System. Sequencing results were compared with known sequences in the GeneBank database using the Basic Local Alignment Search Tool for identification.

2.4. Bacterial strains

The bacterial strains utilized in the study were obtained from the Culture Repository of the Microbiology Laboratory at SIAHs, SUIT, Peshawar. These strains encompassed pathogenic bacteria, specifically Entero-pathogenic *Escherichia coli* (*E. coli*), *Enterococcus* spp., *Salmonella typhi*, and *Staphylococcus aureus* (*S. aureus*), notably including methicillin-resistant *S. aureus*. Prior to the study, these isolates had undergone characterization via standard microbiological procedures and species-specific primers targeting the 16S rRNA gene using conventional Polymerase Chain Reaction (PCR).

2.5. Probiotic bacteria's antagonistic activity against pathogenic enteric bacteria

Antagonistic activity of probiotic bacteria against pathogenic enteric bacteria was assessed via well diffusion method. Pathogenic bacteria were cultured overnight, spread on Mueller Hinton (MH) agar, and plates were air-dried. Sterilized probiotic samples, obtained from MRS broth cultures, were applied onto MH plates. Clear zones around probiotics indicated their efficacy against pathogenic enteric bacteria.

2.6. Well Diffusion Method

The bactericidal activity of *Lactobacillus* and *Bifidobacterium* strains against specific bacteria was assessed using the well-diffusion method. Pathogenic bacteria were cultured in nutritional broth and swabbed onto MH agar plates. Cell-free supernatants from isolated *Lactobacilli* and *Bifidobacteria* were loaded into wells. After 24 hours of incubation at 37°C, inhibitory zones were measured.

2.7. Minimum inhibitory concentration

To assess the antimicrobial properties of isolated *Lactobacillus* and *Bifidobacterium* supernatants, the agar well diffusion method was employed on Mueller-Hinton Agar plates. Wells were created, and probiotic samples were added at concentrations ranging from 10 µl/ml to 100 µl/ml v/v. Bacterial cultures were inoculated and plates were incubated at room temperature for 24 hours. Ciprofloxacin antibiotic discs and sterile distilled water served as positive and negative controls, respectively. Antimicrobial activity was quantified by measuring the diameter of the clear zones around the wells, indicative of the zone of inhibition, to determine the Minimum Inhibitory Concentration (MIC).

3. Results

3.1. Bacterial Culture Isolation and Purification

Following sample processing and overnight incubation, samples were cultured in Nutrient broth for bacterial growth. Isolated samples were preserved in 50% glycerol stock solution for further phenotypic analysis. Gram staining revealed purple colonies with rod-shaped bacteria arranged in chains, indicative of gram-positive bacteria. Both *Lactobacillus* and *Bifidobacterium* strains were confirmed to be gram-positive. Biochemical tests, including the oxidase and catalase tests, were performed. *Lactobacillus* and *Bifidobacterium* strains showed negative results for both oxidase and catalase tests. Additionally, indole testing indicated negative results for indole production. From sample 08, 11, 17, 22, 23, and 48, a total of 6 pure bacterial isolates were obtained. Samples 08, 17, 22, and 23 were from buffalo's milk, while samples 11 and 48 were from cow's milk. These selected isolates were further analyzed through sequencing and phylogenetic analysis to identify specific species.

Table .1. Results of Morphological and Biochemical tests

Isolates No	Gram staining results	Oxidase results	Catalase results	Indole results	Suspected Microbes
8	+	_	_	_	Isolate identified
11	+	_	_	_	Isolate identified
17	+	_	_	_	Isolate identified
22	+	_	_	_	Isolate identified
23	+	_	_	_	Isolate identified
48	+	_	_	_	Isolate identified

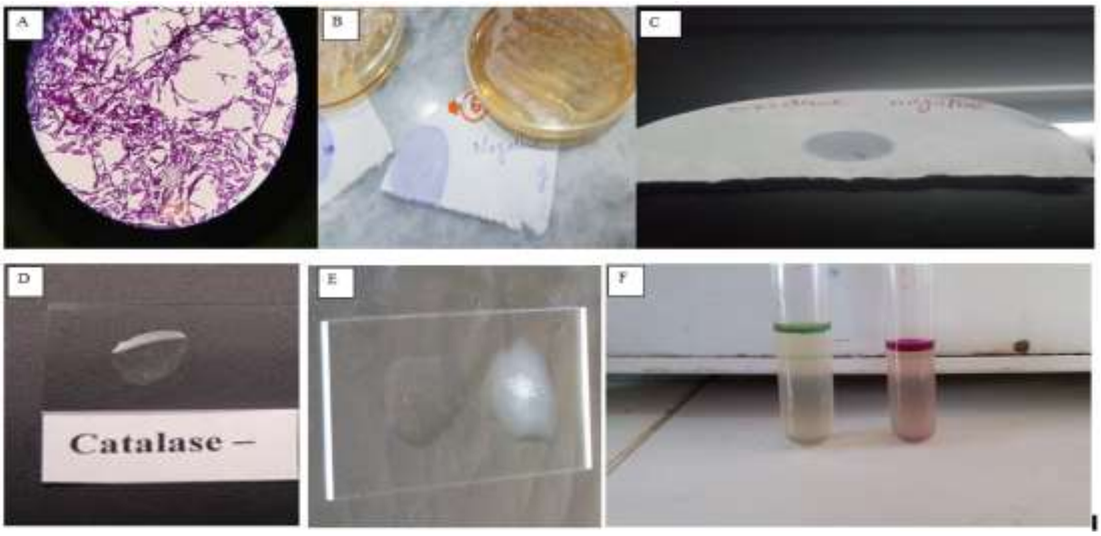

Fig.1: Show the (A) Microscopic picture of purple colonies of *Lactobacillus* and *Bifidobacterium* (B) negative Oxidase test for *Lactobacillus* on a wet filter paper method (C) Oxidase Negative results for *Bifidobacterium* (D) Shows Catalase negative results of *Lactobacillus* (E) Shows Catalase negative results of *Bifidobacterium* and (F) Indole negative results of bacterial culture.

3.2. Sequence and Phylogenetic analysis

The PCR products of 16S rRNA from *Lactobacillus* and *Bifidobacterium* were sequenced, and the obtained sequences were subjected to cleaning. Subsequently, the cleaned sequences were compared with the NCBI gene bank database using BLAST to identify sequence similarities. Phylogenetic trees were constructed using MEGA software based on the sequence data obtained, allowing for the visualization of evolutionary relationships among the isolated Lactobacillus and Bifidobacterium strains.

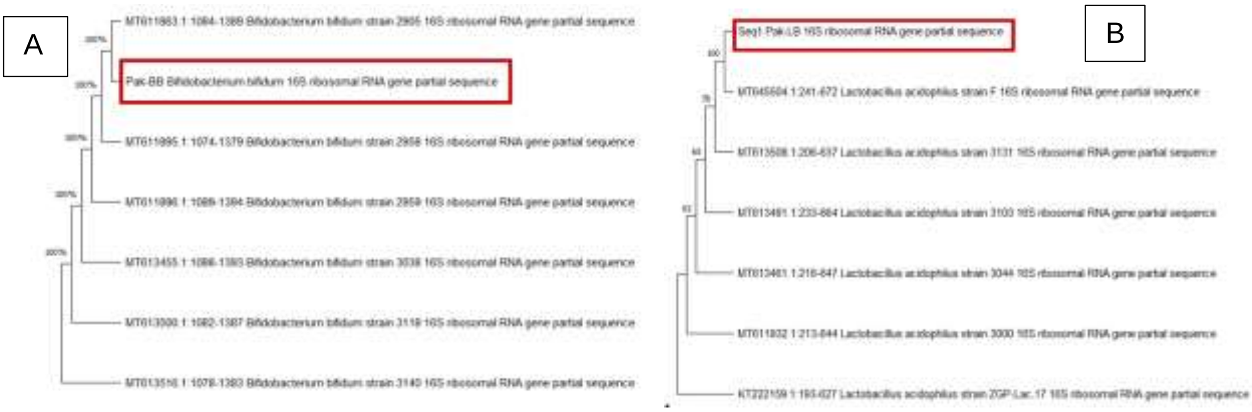

Fig. 2: Phylogenetic tree of (A) *Bifidobacterium bifidum* and *(B) Lactobacillus acidophilus* based on *16S* rRNA gene.

3.3. Lactobacillus and Bifidobacterium isolates exhibited antibacterial activity

Lactobacillus and *Bifidobacterium* strains were tested for their bactericidal activity against enteropathogenic bacteria using the well-diffusion method. The method involved creating wells on agar plates inoculated with the pathogenic bacteria and adding the probiotic samples. After incubation, the extent of bacterial growth inhibition around the wells indicated the bactericidal activity of the probiotic strains.

Table 2. Antibacterial activity of bacterial isolates

Name	Positivecontrol ciproflaxacin (%)	Negative control (%)	*Lactobacillus* zone of inhibition	*Bifido-bacterium* spp zone of inhibition
			mm	Mm
S.aureus	100%	0%	13mm	17mm
E.coli	100%	0%	17mm	20mm
S.typhi	100%	0%	15mm	18mm
Enterococcus	100%	0%	19mm	14mm

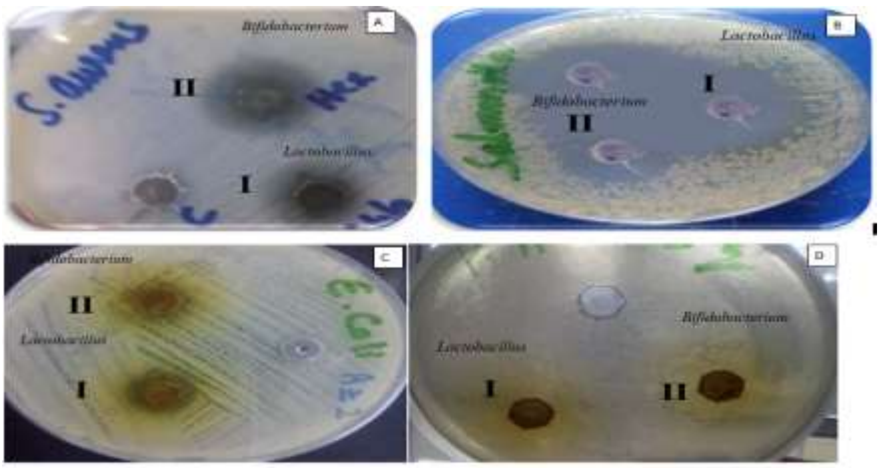

Fig .3: Shows the antibacterial activity *Bifidobacterium* and *Lactobacillus* against (A) *S. aureus* (B) *Sallmonella typi* (C) *E.coli* and (D) *Enterococcus*

3.4. Antibacterial activity was determined via MIC

Bifidobacterium and Lactobacillus exhibited potent antimicrobial activity against the tested isolates, particularly against *S. aureus*. Significant inhibition was observed across a range of concentrations, including 100 µl/ml, 80 µl/ml, 40 µl/ml, 20 µl/ml, and 10 µl/ml. These findings underscore the potential of these probiotic bacteria as natural antimicrobial agents against pathogenic strains.

Table. 3. Antimicrobial activity of *Lactobacillus*

S. No	Bacterial species	Positive control ciproflaxacin (%)	Negative control (%)	*Lactobacillus* zone of inhibition				
				100µl	80µl	40µl	20µl	10µl
1	*S.aureus*	100%	0%	22mm	15mm	10mm		
2	*S.typhi*	100%	0%	20mm	15mm			
3	*Enterococcus*	100%	0%	20mm	14mm			
4	*E.coli*	100%	0%	20mm	15mm			

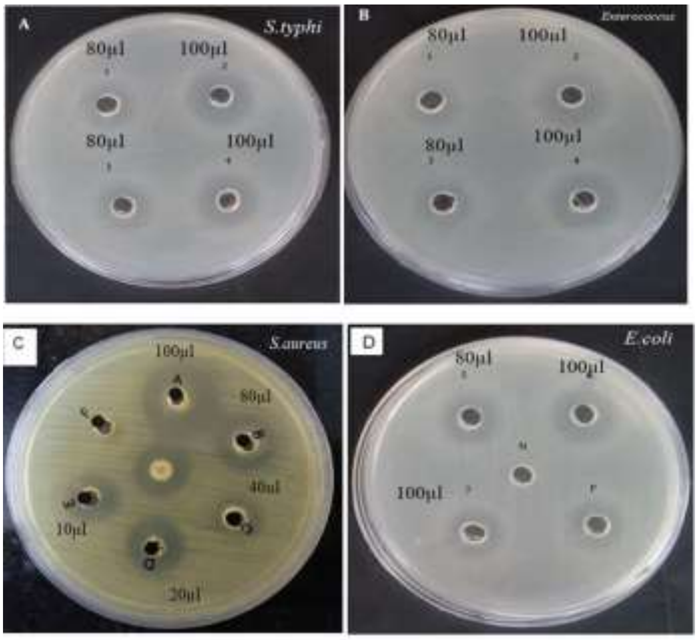

Fig .4: Show the Zone of inhibition against (A) *S.typhi* (B) *Enterococcus (C) S. aureus and (D) E.coli*

4. Discussion

The current study undertook a comprehensive characterization of clinical isolates through standard microbiological procedures, revealing their resistance to various classes of antibiotics. Notably, *Lactobacillus* and *Bifidobacterium* isolates demonstrated efficacy against both Gram-positive and Gram-negative pathogens, encompassing *Escherichia coli*, *Salmonella* species, *Staphylococcus aureus (S. aureus),* and *Enterococcus*. The global dissemination of infectious diseases caused by bacterial pathogens presents a significant threat to public health. Our investigation unveiled the inhibitory and lethal effects of extracts against these pathogenic bacteria, with discernible zones of inhibition observed against *E. coli, Salmonella* species, *S. aureus,* and *Enterococcus*. In line with our findings, a study reported similar inhibitory activity against *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae,* and *Salmonella paratyphi* A, with inhibition zones ranging between 12 and 32 millimeters (Britto, Sebastian, & Sujin, 2012). Probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium* are extensively utilized for various biological purposes. These include the production of secondary metabolites with potential antimicrobial, antioxidant, and other biological properties by lactic acid bacteria (LAB), as highlighted in studies by (Gomes & Malcata, 1999; Kailasapathy & Chin, 2000; Ku, Park, Ji, & You, 2016). Furthermore, investigated the antimicrobial activity of *Lactobacillus* strains against five diarrheagenic *E. coli* pathotypes, revealing mild inhibitory activity against these pathogens, consistent with our findings of mild activity against *E. coli* but effectiveness against Gram-positive pathogens (Liévin-Le Moal & Servin, 2014; Vila et al., 2016).

Similarly study reported significant inhibitory effects against carbapenem-resistant *E. coli*, aligning with our observations of substantial inhibition against *E. coli* isolates (Aunins, Erickson, & Chatterjee, 2020). Furthermore, in vitro studies have demonstrated the antibiotic activity of certain *Lactobacillus* strains against various pathogens including *Clostridium difficile*, *E. coli*, *Shigella* spp., *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *S. aureus*, consistent with our investigation (Chen, Lai, Toh, & Tang, 2019; Darbandi et al., 2022; Liévin-Le Moal & Servin, 2014). Additionally, explored the probiotic potential of *Lactobacillus* strains isolated from traditional yogurt, focusing on their effectiveness in the human vaginal system (Arshad, Mehmood, Hussain, Khan, & Khan, 2018; Nami, Haghshenas, & Khosroushahi, 2018). The antibacterial activity of probiotics, particularly *Lactobacillus*, is a critical criterion for their selection. Probiotics exert antimicrobial properties through the production of various compounds such as organic acids, hydrogen peroxide, and bacteriocins. A study reported the antibacterial effects of *Lactobacillus* isolates against indicator microorganisms including *S. aureus*, *Enterococcus faecalis*, *E. coli*, *Salmonella typhii*, and native isolated *Shigella* spp., aligning with our study findings (Mojgani, Hussaini, & Vaseji, 2015; Sornsenee, Singkhamanan, Sangkhathat, Saengsuwan, & Romyasamit, 2021).

5. Conclusion

Probiotics such as *Lactobacillus* and *Bifidobacterium*, derived from yogurt, demonstrate efficacy against diverse pathogenic strains, supporting digestion, vitamin absorption, and immune function. Lactobacillus, notably abundant in fermented foods like yogurt, aids in managing conditions like diarrhea, IBS, and *H. pylori* infections. Consumption of yogurt containing these probiotics is recommended for health benefits. Certain strains of *Lactobacillus*, such as *L. acidophilus*, offer additional benefits against vaginal yeast infections. Scientific research highlights the efficacy of *Lactobacillus* or *Bifidobacterium* supplements and specific strains found in yogurt or milk to enhance the body's defense mechanisms. Incorporating probiotic-rich foods like yogurt into one's diet can contribute to overall health and wellbeing, offering a natural approach to support gut health and immunity.

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