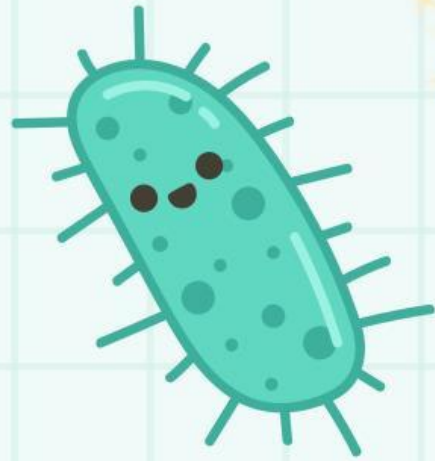




# Student Worksheet

Identification of Probiotic  
Bacteria Kinship Relationships

For 10th grade students



# STUDENT WORKSHEET

**Theme** : **Bacteria**  
**Sub-theme** : **The Role and Classification of Bacteria**  
**Class** : **X**  
**Semester** : .....  
**Group** : .....  
**Group Members** : **1**.....  
**2**.....  
**3**.....  
**4**.....  
**5**.....

## Learning Outcomes

- To learn how to use MEGA software for conducting phylogenetic analysis based on bacterial 16S rRNA gene sequences.
- To identify probiotic bacterial species present in the intestinal tract of Sinyonya common carp (*Cyprinus carpio*) through morphological, biochemical, and molecular data.
- To retrieve and utilize reference bacterial sequences from the NCBI GenBank database for bioinformatics analysis.
- To perform multiple sequence alignment to determine sequence similarity and homology among bacterial species.
- To construct and interpret a phylogenetic tree to understand the evolutionary relationships between probiotic bacterial isolates and reference species.

# ACTIVITY

## A. Introduction

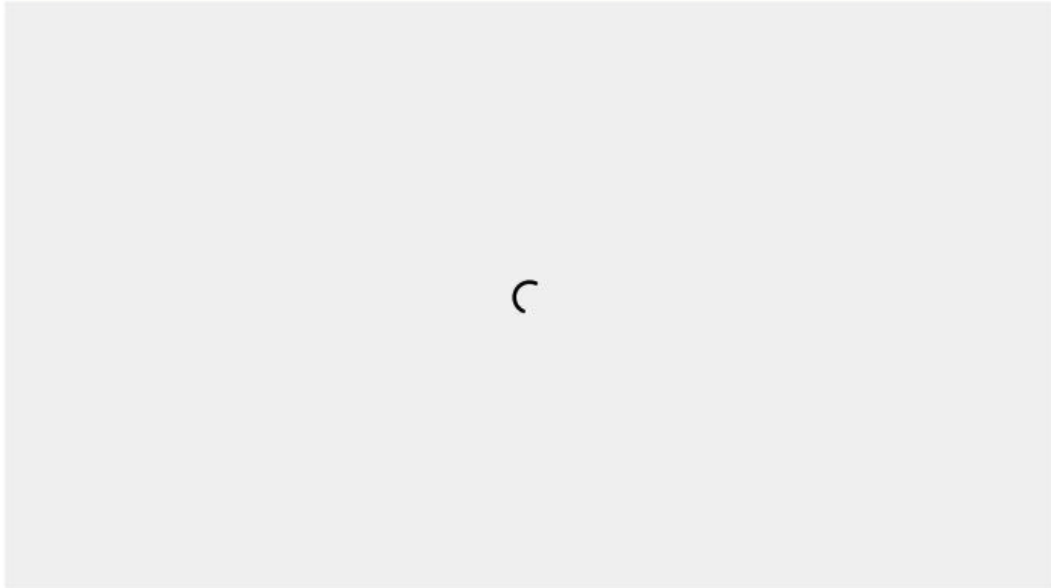
Answer the following questions!

| Question                                                                      | Expected Answer |
|-------------------------------------------------------------------------------|-----------------|
| What do you know about bacteria?                                              |                 |
| What types of bacteria do you know?                                           |                 |
| What are the roles of bacteria that you know?                                 |                 |
| In your opinion, can probiotic bacteria provide benefits to living organisms? |                 |
| What is a phylogenetic tree?                                                  |                 |
| What do you know about NCBI?                                                  |                 |
| What do you know about MEGA?                                                  |                 |

**Now, let's watch a video about how probiotic bacteria work and how bacteria work in digestion!**

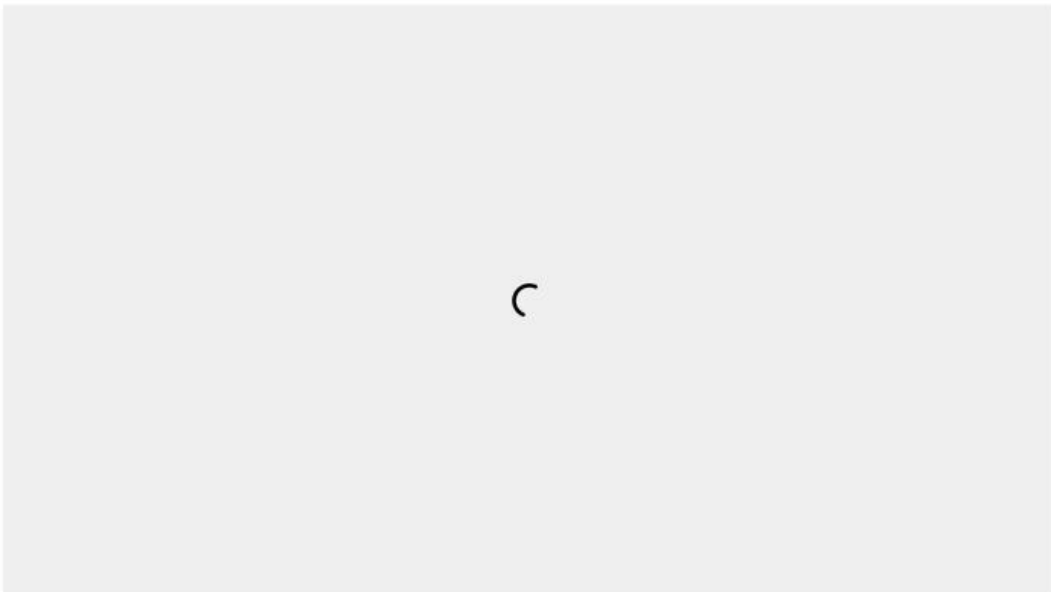
**A. How do Probiotic Bacteria Work?**

<https://www.youtube.com/watch?v=ZSSWQVPLUMw>



**B. How do Bacteria Work in Digestion?**

<https://www.youtube.com/watch?v=laUPud5PVhU>





## B. Investigation

In Pandeglang, Banten, there is a rare endemic fish species with high economic value, known as Sinyonya Goldfish (*Cyprinus carpio*). This fish is recognized for its golden-yellow body and distinct narrow eyes. In addition to its potential as an ornamental and food fish, the Sinyonya Goldfish also harbors a diverse community of microbes in its digestive tract, including probiotic bacteria.

A researcher has carried out a series of isolation and identification procedures of probiotic bacteria from the intestinal organs (stomach and intestine) of Sinyonya Goldfish. The steps performed include:

1. Sampling intestinal organs from fish aged approximately 2 months at a hatchery.
2. Bacterial isolation using selective de Man Rogosa and Sharpe Agar (MRS Agar) and the pour plate method.
3. Purification of colonies using the quadrant streak technique to obtain pure colonies.
4. Observation of colony morphology (color, shape, margin, surface).
5. Gram staining to determine cell wall characteristics (Gram-positive or Gram-negative).
6. Catalase test to detect the presence of catalase enzyme.
7. Proteolytic test to determine the ability to produce protease enzymes.

From the identification results, the researcher found that one isolate showed characteristics of Gram-positive bacteria, rod-shaped, non-motile, catalase-negative, and proteolytic. These traits indicate the genus ***Lactobacillus***, which is widely known as a probiotic bacterium.

*To understand the relationship by constructing a phylogenetic tree, let's help researchers compile it!*



**Let's help researcher with the steps below!**

However, to confirm its identity and determine its phylogenetic relationship with other species, the researcher needs to perform a phylogenetic analysis based on the molecular data. This analysis can be conducted using the **MEGA** (*Molecular Evolutionary Genetics Analysis*) software.

Now, let's help the researcher to construct the phylogenetic tree of the isolated bacterium. The data used will consist of the DNA sequence obtained from PCR amplification of the isolate and reference sequences from the **NCBI** database.

### **Probiotic Bacteria**

- *Lactobacillus gasseri* 16S rRNA
- *Lactobacillus casei* 16S rRNA
- *Lactobacillus fermentum* 16S rRNA
- *Lactobacillus acidophilus* 16S rRNA
- *Bacillus subtilis* 16S rRNA (outgroup/distant comparison)

### **Bacterial Sample Sequence**

Accessible via the link or barcode below!

<https://bit.ly/fasta-sequences>



**Scan Here!**



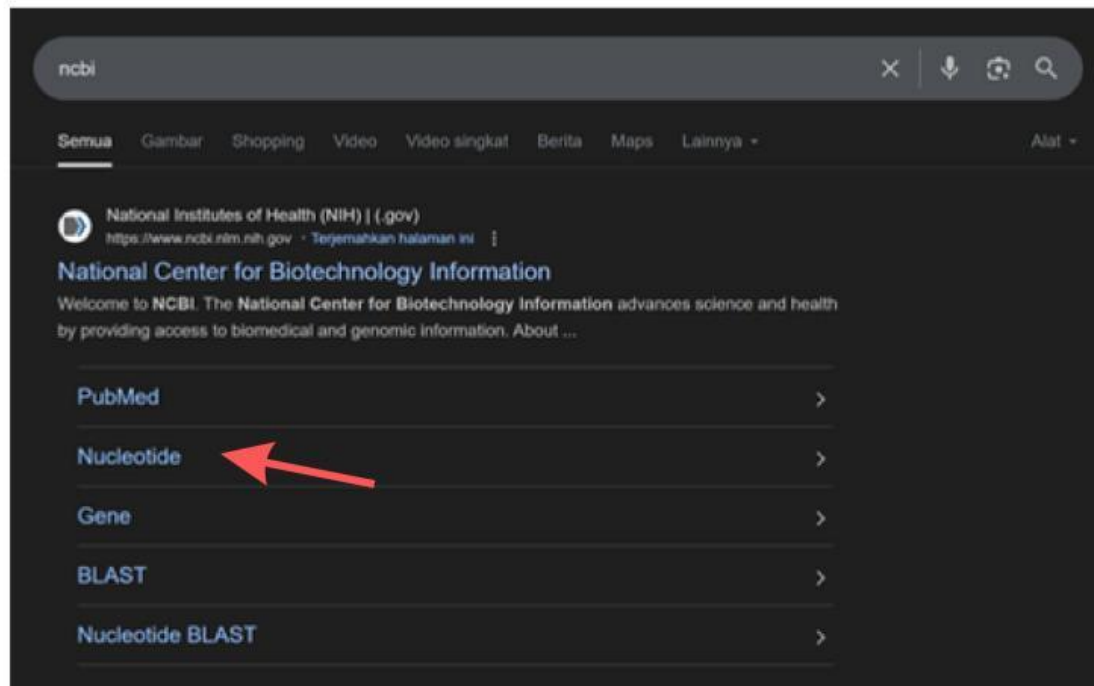
**Let's start building a phylogenetic tree with the following steps!**



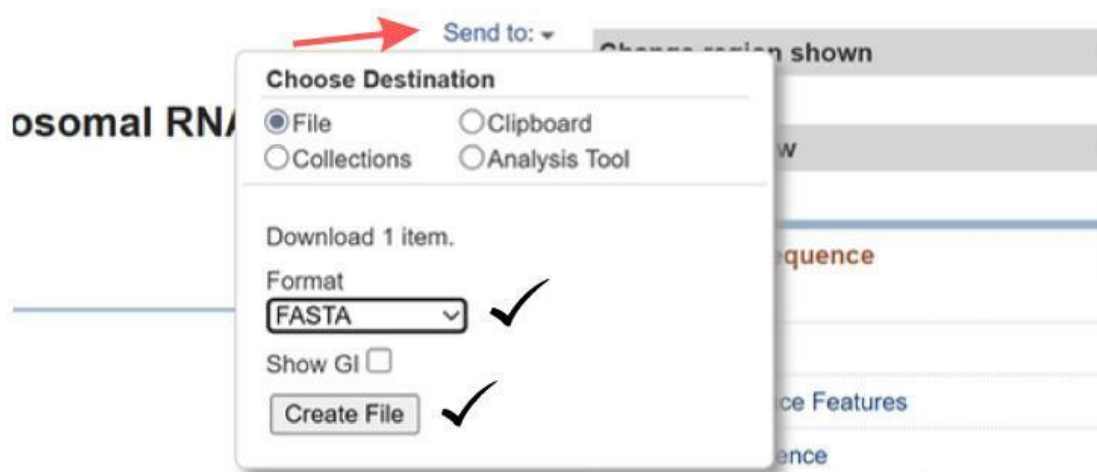


## Obtaining Sequence Data from NCBI

- Go to **NCBI** website, click **Nucleotide**.



- Type the target species name, for example:
  - Lactobacillus gasseri* 16S rRNA
  - Lactobacillus casei* 16S rRNA
  - Lactobacillus fermentum* 16S rRNA
  - Lactobacillus acidophilus* 16S rRNA
  - Bacillus subtilis* 16S rRNA (outgroup/distant comparison)

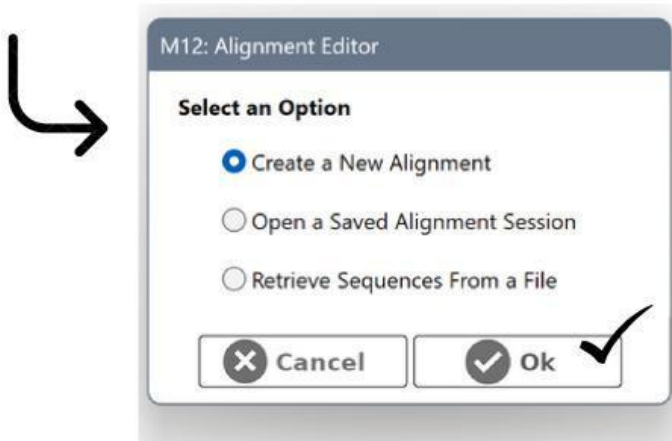
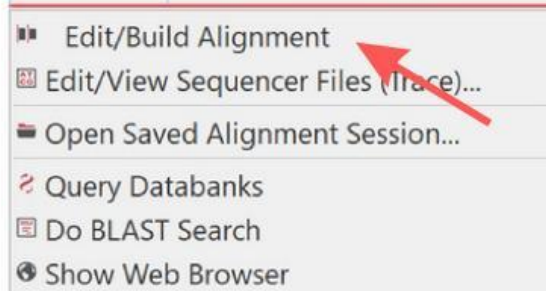


- Select the appropriate record, click FASTA, copy the sequence, and save it as a .fasta file.



## Creating Alignment in MEGA

- Open MEGA → Align → Edit/Build Alignment → Create New Alignment → select DNA.

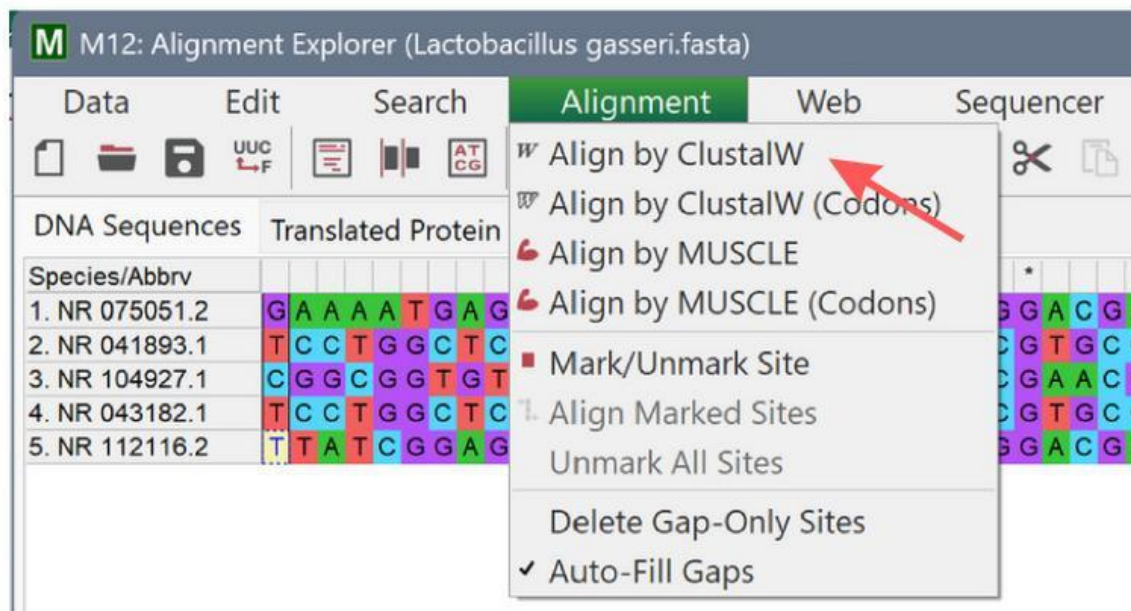
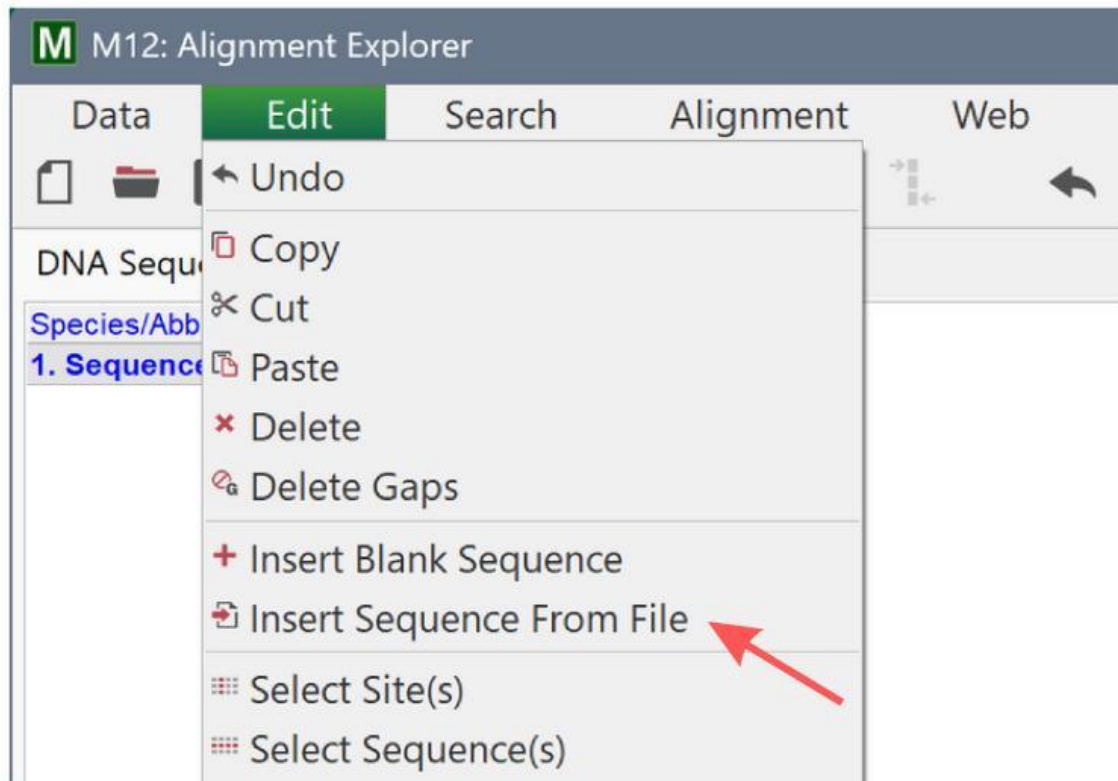






## Creating Alignment in MEGA

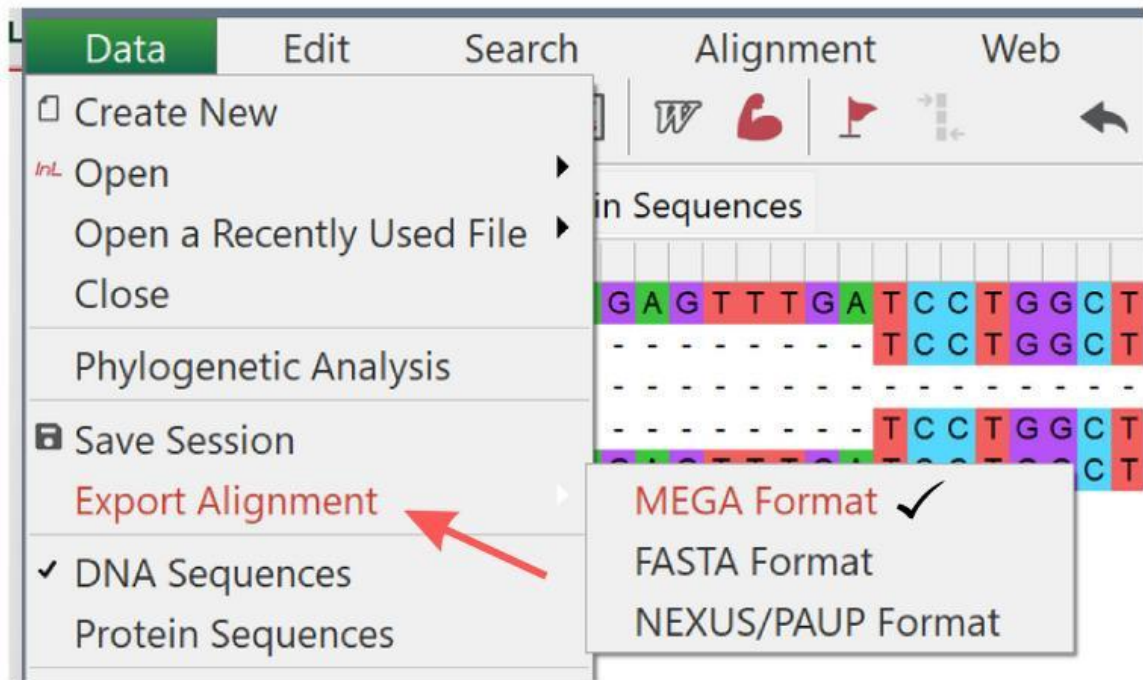
- Import the combined .fasta file. → Perform alignment using ClustalW → Save the aligned file as .meg.





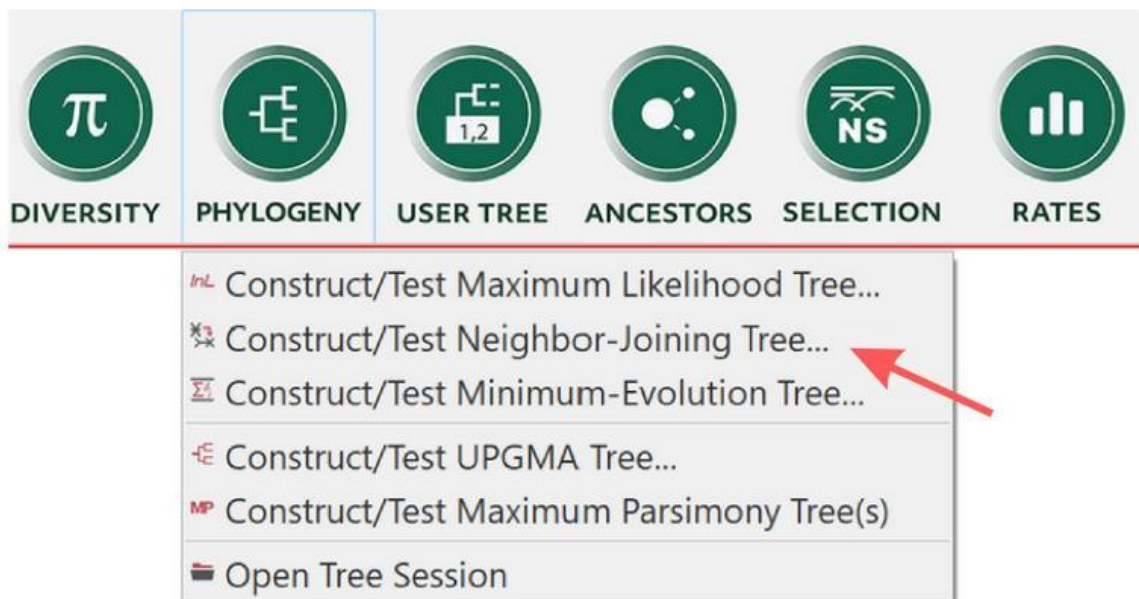
## Creating Alignment in MEGA

- Save the aligned file as .meg.



## Building the Phylogenetic Tree

- From the aligned file, select Phylogeny → Construct/Test Neighbor-Joining Tree.





## Building the Phylogenetic Tree

- Choose a nucleotide substitution model, such as Tamura-Nei model.
- Enable the Bootstrap test (e.g., 1000 replications) to assess branch reliability.
- Run the analysis and wait for the tree to be generated.

| RATES AND PATTERNS                                               |                    |
|------------------------------------------------------------------|--------------------|
| Rates among Sites →                                              | Uniform Rates      |
| Pattern among Lineages →                                         | Same (Homogeneous) |
| DATA SUBSET TO USE                                               |                    |
| Gaps/Missing Data →                                              | Pairwise deletion  |
| SYSTEM RESOURCE USAGE                                            |                    |
| Number of Threads →                                              | 7                  |
| <div><span>Help</span> <span>Cancel</span> <span>Ok</span></div> |                    |

- After clicking OK, Mega will construct a phylogenetic tree based on the Fasta file that has been entered, and the phylogenetic tree will be complete.



**Congratulations!**  
**You have helped**  
**researcher build a**  
**phylogenetic tree!**



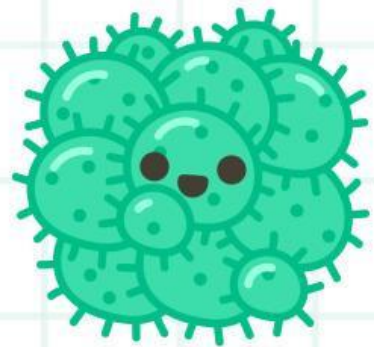
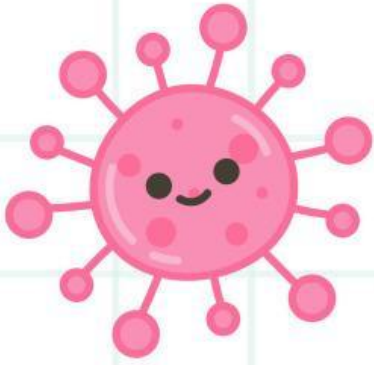
## Now, let's answer the questions below!

Based on the phylogenetic tree analysis you have created, answer the questions below

| Question                                                                                                                                                                          | Expected Answer |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|
| Does the bacterial sample from the research show a close relationship with probiotic bacteria from the genus <i>Lactobacillus</i> ? Explain your answer.                          |                 |
| Based on the phylogenetic tree, is the bacterial sample more closely related to <i>Lactobacillus gasseri</i> or <i>Lactobacillus casei</i> ? Provide evidence from your analysis. |                 |
| According to your analysis, to which genus does the bacterial sample belong? Explain the characteristics that support this identification.                                        |                 |
| Explain how phylogenetic tree analysis can help researcher confirm the identity of probiotic bacteria isolated from the intestine of Sinyonya Goldfish.                           |                 |

**Please answer the questions below.**

| Question                                                                      | Expected Answer |
|-------------------------------------------------------------------------------|-----------------|
| What do you know about bacteria?                                              |                 |
| What types of bacteria do you know?                                           |                 |
| What are the roles of bacteria that you know?                                 |                 |
| In your opinion, can probiotic bacteria provide benefits to living organisms? |                 |
| What is a phylogenetic tree?                                                  |                 |
| What do you know about NCBI?                                                  |                 |
| What do you know about MEGA?                                                  |                 |



**Congratulations!**  
**You have completed**  
**today's lesson!**

