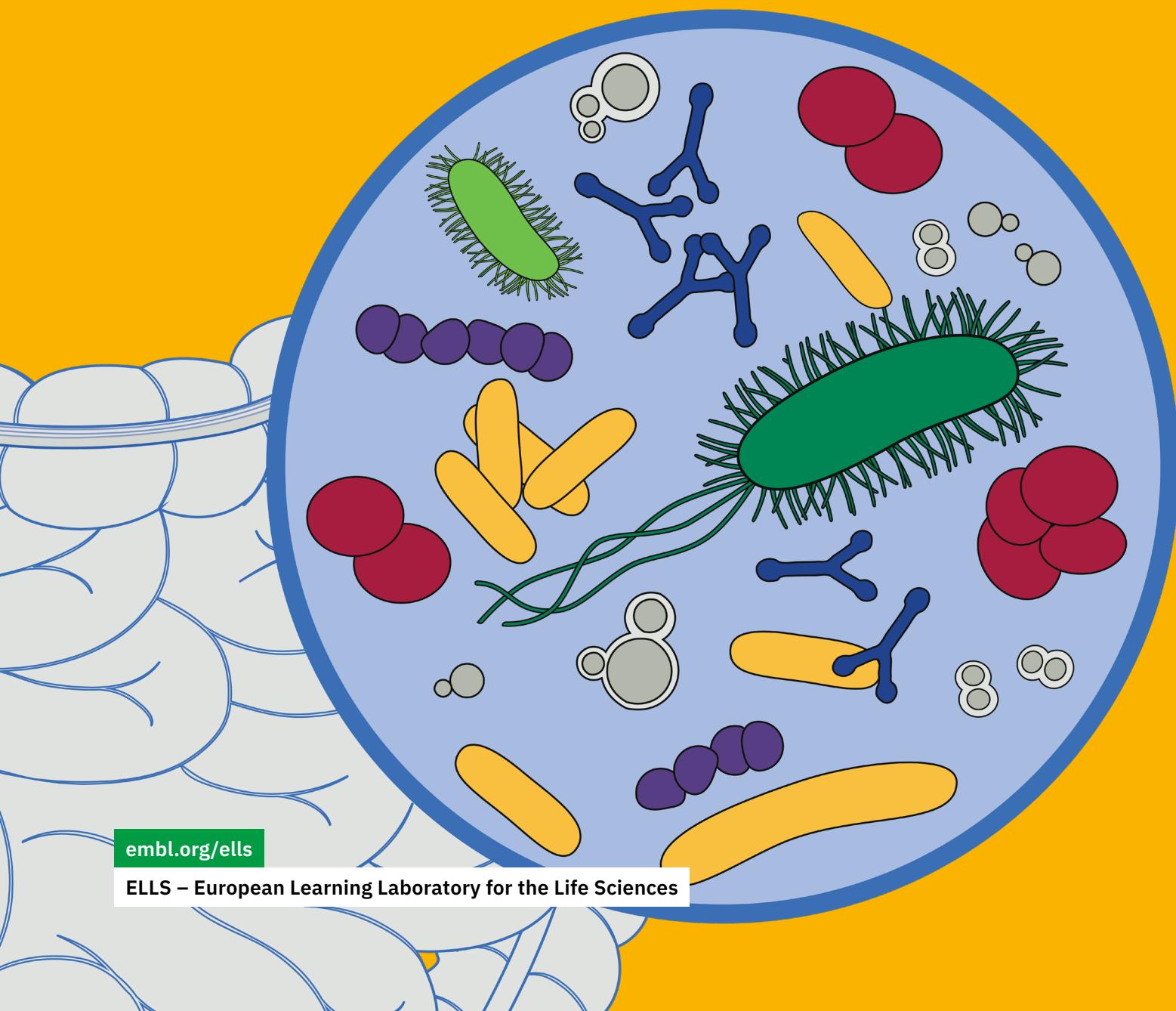




# Introducing your microbiome

The educational resource package



## About EMBL

EMBL is Europe's leading laboratory for the life sciences. We are an intergovernmental organisation supported by over 25 member states and operating across six sites in Europe.

EMBL performs fundamental research in molecular biology, studying the story of life. Our research drives the development of new technology and methods in the life sciences, and we work to transfer this knowledge for the benefit of society.

## About ELLS

The European Learning Laboratory for the Life Sciences (ELLS) is EMBL's science education department. We share the scientific discoveries of EMBL through inspiring learning and outreach experiences.

Our activities are open to European school science teachers, and to young people of all backgrounds aged between 10 and 19 years.

The ELLS programmes convey complex, cutting-edge topics in life science research in an exciting and insightful way, fostering the discovery of current research trends, the scientific method, and scientific career paths. Our activities are developed and run in close collaboration with EMBL scientists.

Visit our website for further information about our activities: [embl.org/ells](http://embl.org/ells)

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# Information for teachers

## Introducing your microbiome

The educational resource package



# Information for teachers

## Introduction

The microbiome research field is developing at a very fast pace unravelling more and more interesting facts about the microbes that live in and on us. Understanding the impact of their presence and interactions with their hosts has the potential to impact future developments in various areas of our lives, from ecology and agriculture to medicine.

Therefore, this teaching resource seeks to challenge students to better understand the finely balanced relationship between the presence of certain microorganisms in our gut and their impact on an individual's health. To this end, they will work with various means for classification such as dichotomous keys, relatedness matrices, cladograms and phylogenetic trees.

## Overview

This teaching resource is aimed for use by secondary school teachers with students aged 14-19. The resource package is considered for face-to-face learning. It can, however, easily be implemented into virtual classrooms as well. The resource package is composed of a short animation video (<https://www.youtube.com/watch?v=OisAq1zRo-M>) and five activities.

The implementation of the whole resource package might take up to 6 school hours. However, all activities can be used independently of each other or in flexible combinations of several activities. This allows for maximal flexibility and eases the implementation in class.

Exercises 1 to 4 do not require any particular equipment. Exercise 5 is computer-based and comprises the use of the online tool "Interactive Tree of Life" (iTOL) (<https://itol.embl.de/>). In case no computers are available to use iTOL, Activity 4 is provided as a paper-based alternative to help students apply their theoretical knowledge on cladistics. However, Activity 4 can also be used to introduce students to analysing phylogenetic trees before moving on to using iTOL.

To equip students with the information they need to complete the exercises, **Fact sheets** on the microbes mentioned in the activities as well as phylogenetic trees are provided. The different flash cards are organised on separate pages of a PDF file, giving the teachers the opportunity to choose which ones to hand out to their students. Teachers might also ask more advanced students to prepare topics they are familiar with and share them with their classmates.

As part of the resource package, an **iTOL basics guide** is included, step-by-step depicting the work flow with the online tool.

Please note that the time we propose is needed to conduct the activities is a rough estimate and strongly depends on factors like the students' prior knowledge and the way teachers choose to implement the respective activities in their classes.

## Learning outcomes

The students will

- identify microorganisms involved in various disease states in the gut;
- appreciate the importance of the microbiome in health and disease;
- learn how computers use sequence data to build phylogenetic trees;
- understand how phylogenetic trees are built and analysed.

## Structure of the resource

The resource contains five main activities with increasing complexity.

### Activity 1

In **Activity 1**, students are given information on the health status and recent activities of various individuals along with the composition of their gut microbiome. By referring to fact sheets that contain information on individual microorganisms, students are asked to identify which microbiome belongs to which individual. For example, in the fact sheets, students will find that *Clostridioides difficile* synthesizes a toxin which causes watery diarrhoea in patients, prompting them to link a microbiome with an increased abundance of *C. difficile* to someone with symptoms of diarrhoea.

Estimated time needed: 20 min.

### Activity 2

In **Activity 2**, students are asked to complete a flowchart resembling a dichotomous key. The activity allows students to use a combination of unique characteristics to classify organisms. This particular dichotomous key is referring to six microorganisms: *Methanobrevibacter smithii*, *Campylobacter jejuni*, *Vibrio cholerae*, *Clostridioides difficile*, *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. As an example, among these microorganisms, only *M. smithii* is able to generate methane gas as a result of cellular respiration. This feature allows students to separate this archaeon from the rest of the organisms and to classify it. Activity 2 also contains questions that aim to set the scene for Activity 3 and 4, where members of the microbiome are classified based on DNA sequencing information rather than biochemical and physical characteristics.

Estimated time needed: 30 min.

### Activity 3

In **Activity 3**, students learn how computers use DNA sequence information to build relatedness matrices through which phylogenetic trees are created. A relatedness matrix is created by using simple mathematical operations, where the mismatches between two sequences are divided by the total nucleotide number of the respective sequence.

Estimated time needed: 30 min.

### Activity 4

In **Activity 4**, students practice their tree-analysing skills by answering questions related to a cladogram that shows the evolutionary relationship between microorganisms from a healthy human gut. Here, students identify nodes representing common ancestors, branches identifying individual sequences/organisms and infer relatedness based on sequence information.

Estimated time needed: 40 min.

### Activity 5

In **Activity 5**, students learn to use a web tool, the interactive Tree of Life (iTOL) to construct phylogenetic trees using sequence information. Here, phylogenetic trees showing the microbial composition of different individuals can be built and modified. iTOL's ability to provide a variety of alternatives in displaying trees allows students to get an authentic interaction with this bioinformatics tool.

Estimated time needed: 60-90 min.

The activity worksheet is designed to give the students the opportunity to note their results.

## Links to the curriculum

Curriculum	Links
AP	7.9
IB	5.3, 5.4, B.5
NGSS	HS-LS4-1
GCSE	4.6.3, 4.6.4

## Prerequisites and prior knowledge

Prior knowledge in topics such as cells and cellular processes, classification of organisms, classification and characterisation of bacteria as well as basic knowledge of organic molecules is required.

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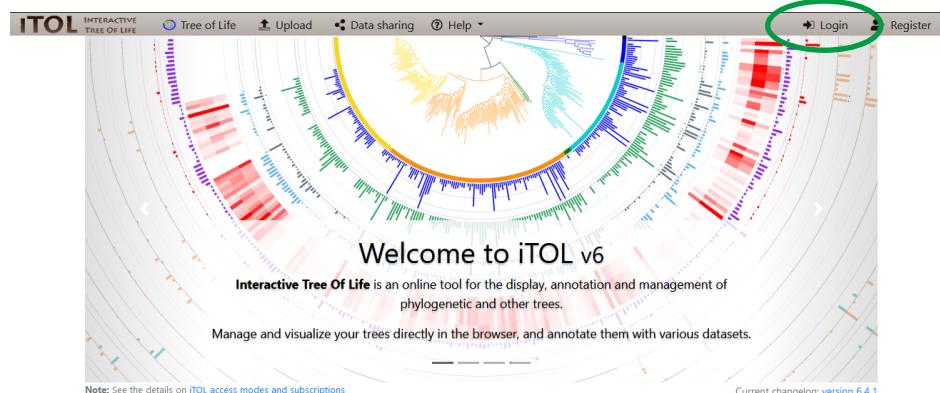
## Disclaimer for external links

Links to non-EMBL websites are being provided as a convenience and for informational purposes only. They do not constitute an endorsement or an approval by EMBL. EMBL has no affiliation with the content owners of those external sites and bears no responsibility for the accuracy, legality or content of the external sites or for that of subsequent links.

## Login information for iTOL

For the duration of the course, we have created an account on the Interactive Tree of Life (iTOL) website. Connected to this account is a complementary standard subscription for iTOL for the duration of the course.

To log in, please visit the iTOL website at <https://itol.embl.de/> and go to “Login” on the top right hand corner.



Log in using the following data:

**Login:** ELLS

**Password:** TeacherTraining21

## After the course

After the course, you can continue to use iTOL with a free account. Just click "Register" on the top right hand corner and register your new account. That way, you will have all iTOL annotation features fully accessible, however, you will not be able to save the trees. For more information on access, please visit: <https://itol.embl.de/infoReg.cgi?f=p>

If you would like to run this activity with your students, you can request complimentary standard access for the duration of the assignment (see: <https://itol.embl.de/pricing.cgi>).

# Animation transcript

## Introducing your microbiome

A tale of two guts



# Introducing your microbiome

## A tale of two guts

### Teachers' note

The animation “Introducing your microbiome: a tale of two guts” introduces students to the human gut microbiome in a playful way using 2D illustrations and 3D stop-motion. The story covers some key aspects of the microbiome in health and disease such as microbiome diversity, introduces several important microbial species and illustrates the link between scientific knowledge and research.

Alongside the animation, we provide you with a short teaser text of the animation and the transcript of the voice-over.

### Animation

Length: 9:38 minutes

<https://www.youtube.com/watch?v=OisAq1zRo-M>

### Teaser

We join two friends, Laura and Kathryn, on a journey into a whole new world. A world which we cannot see, but exists within all of us - the microbiome. On this journey, we come face to face with some key inhabitants of Laura and Kathryn's gut microbiomes. The various species of microorganisms that call their bodies home result in the two friends having very different experiences and learning some important lessons along the way.

### Copyright

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## Transcript

Time stamp	Transcript
0:07	Meet Kathryn. Kathryn is someone who you would describe as healthy - she gets regular exercise, plenty of sleep, drinks lots of water, and eats a well balanced diet which gives her all the nutrients she needs.
0:24	Everyone who meets Kathryn can see the benefits of her healthy lifestyle. She is full of energy, rarely gets sick, and is almost always in a good mood. What people don't see, however, are the many tiny inhabitants of Kathryn's body that work to keep her healthy. It turns out that Kathryn, like the rest of us, is not alone.
0:48	This is Kathryn's gut microbiome. A microbiome consists of trillions of microorganisms that call our bodies home. Different parts of our body have different types of microbial tenants, some of whom are allies, and some who we would like to evict, if we could. Let's take a closer look at some of the species of microorganisms that inhabit Kathryn's gut.
1:14	First up is <i>Lactobacillus acidophilus</i> . It is sometimes called <i>L. acidophilus</i> or simply <i>Acidophilus</i> . Thousands of these little fellows live in Kathryn's intestines.
1:27	<i>Acidophilus</i> is a type of bacterium that produces an enzyme called lactase. Lactase breaks down lactose, which is a sugar found in milk and milk products, into lactic acid.
1:41	These friendly bacteria can be found in fermented foods such as yogurt, cheese and Sauerkraut. Because of them, Kathryn gets to enjoy the benefits of reduced cholesterol, prevention of diarrhoea, and a healthier gut overall.
1:57	The next member of Kathryn's microbiome is another gut-friendly bacterium called <i>Lactobacillus bulgaricus</i> .
2:05	These bacteria are used in the production of yogurt. Some of the benefits of <i>L. bulgaricus</i> are managing health conditions such as liver disease, tooth decay, and the common cold. Anyone who's met Kathryn will tell you that she has a great smile and almost never catches a cold. Now we know who she should be thanking for it.
2:26	Bacteria are not the only group of organisms that make up the microbiome. This next inhabitant of Kathryn's gut comes from a group called archaea, and goes by the name of <i>Methanobrevibacter smithii</i> .
2:41	<i>Methanobrevibacter smithii</i> are responsible for digesting complex sugars called polysaccharides. They also remove hydrogen in the gut. Too much hydrogen makes the process of breaking down food by microbes less effective, meaning that the body gets less energy out of the nutrients taken in. So, these archaea ensure that Kathryn gets the most energy she can out of her meals, keeping her fit and healthy.
3:12	This tentacled fellow is one you've probably heard of - <i>Escherichia coli</i> , or <i>E. coli</i> for short. <i>E. coli</i> is part of the normal gut microbiome and is present shortly after birth.

## Transcript - continued

Time stamp	Transcript
3:24	Most strains of <i>E. coli</i> are harmless. They are helpful, even, because they produce vitamin K2 and prevent disease-causing bacteria from occupying the gut. Some strains of <i>E. coli</i> , however, can cause disease themselves, and might give you severe inflammation of the intestines. This is why Kathryn always watches what she consumes, as the harmful strains of <i>E. coli</i> are often found in raw meat and contaminated water.
3:54	Scientists have found that everyone has a microbiome that is unique to themselves. You could even say that your microbiome can identify you much like your fingerprints would. But how does that work?
4:08	There are many factors that influence the composition of your microbiome.
4:13	Factors such as diet, infections, age, stress, physical activity (or the absence of it) and medication all determine the species of microorganisms that live in your gut.
4:27	This is Kathryn's friend Laura. Laura just walked into a doctor's clinic in agony. She has been having severe diarrhoea and muscle cramps.
4:38	Just like Kathryn, Laura is usually healthy, and makes lifestyle choices that foster the growth of a healthy microbiome. However, she recently travelled to an area with very poor sanitation and doing so has brought her these unpleasant symptoms. But who exactly is responsible for this, at the microscopic level?
5:00	The first contender would be <i>Clostridioides difficile</i> , or <i>C. difficile</i> . This species of spore-forming bacteria can be found in the gut in a non-toxic form. However, in situations where the composition of gut microbes changes, for example after taking a course of antibiotics, <i>C. difficile</i> can cause infections such as severe diarrhoea.
5:26	<i>C. difficile</i> is prevalent in soil, faeces, sewage, animals and infected humans. These fellows are a hardy bunch, being able to survive on almost all kinds of surfaces for long periods of time.
5:42	Certain forms of these bacteria produce two different toxins which are responsible for some nasty symptoms such as diarrhoea and cramps. It's a good thing Laura went to the doctor early because a <i>C. difficile</i> infection can be life threatening.
5:59	Another microbe that might be responsible for causing Laura's symptoms is <i>Vibrio cholerae</i> . <i>Vibrio</i> is absent in healthy individuals, but can be found in the gut during infection. These bacteria produce the Cholera toxin which leads to a massive loss of water from the intestine, resulting in secretory diarrhoea, dehydration, and blood pressure dropping to dangerously low levels.
6:25	Cholera is linked to contaminated water. The bacteria responsible are transmitted due to poor sanitation. We can hypothesise that if poor Laura has been infected, she must have had a drink from a contaminated source.

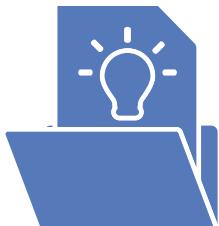
## Transcript - continued

Time stamp	Transcript
6:40	There is another species of bacteria that could have caused Laura's illness. Like <i>Vibrio</i> , <i>Campylobacter jejuni</i> is absent in healthy individuals. It is one of the most common causes of foodborne illnesses worldwide. These bacteria grow inside the cells of the intestine, causing widespread damage to them.
7:02	The most common way to get infected with <i>Campylobacter jejuni</i> is consuming undercooked poultry, and contaminated water or milk. Getting infected with these bacteria could mean suffering abdominal pain and bloody diarrhoea.
7:19	At this point you might be wondering how scientists figure out which microorganisms inhabit a person's microbiome. Enter the world of bioinformatics.
7:30	Bioinformatics is much more than just biology. It combines biology with computer science, information engineering, mathematics and statistics and uses all of these to analyse biological data. Bioinformatics has been responsible for major leaps in the life sciences, such as mapping out the entire human genome.
7:52	The microbiome is an incredibly diverse community, with a huge variety of bacteria, archaea, fungi and viruses.
8:01	Using bioinformatics techniques, scientists are able to zoom in to the genetic code of our microbial neighbours.
8:08	They can zoom in so far that they are even able to tell what the genetic code of the tiny organisms look like. This lets them pinpoint the exact species of microbes that inhabit our bodies...
8:21	...and eventually paint a picture of life within us, giving us a new understanding of how we really function.
8:28	This is all thanks to leaps in technology that come from a combination of different fields of knowledge. The more we work together, the more breakthroughs we are able to make. This is especially true in our current era of science and research.
8:44	It looks like our scientist has finally figured out what's causing Kathryn's friend Laura all this trouble. It turns out it was <i>Vibrio cholerae</i> . Now that she has an answer to her predicament, Laura can carry on with the necessary treatment and will be better in no time!
9:00	The ancient Greek philosopher Aristotle once wrote: The more you know, the more you realise you don't know. This is one of the great paradoxes of science and still holds true to this day. Perhaps even more so than ever before. Laura and Kathryn have embarked on their own journeys to understand the inner workings of their bodies and the myriad of organisms that inhabit them. Who knows what they will find along the way?

# Fact sheets

## Introducing your microbiome

The educational resource package



## Introducing microbes that can be found in the gut -part 1

*Lactobacillus acidophilus & Lactobacillus bulgaricus* are rod-shaped and immobile Gram-positive bacteria. In order to produce energy, they ferment available glucose molecules in the gut and create lactic acid as a by-product.

**Healthy gut:** normally found in the gut.

**Sources:** food such as yogurt, kimchi, tempeh and other fermented food

**Function:** facilitate digestion, maintain an acidic environment and compete with pathogenic microbes.

**Health impact:** typically used in probiotics to restore balance in the gut flora.



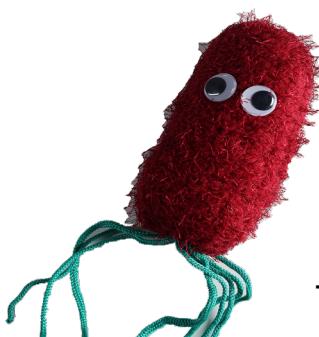
*Methanobrevibacter smithii* is a rod-shaped archaeon. In order to produce energy necessary for survival, this organism uses bacterial waste products such as CO<sub>2</sub> and H<sub>2</sub> gas to produce methane (CH<sub>4</sub>) gas. This process is unique to a group of archaea called the methanogens.

**Healthy gut:** the predominant archaeon in the human gut.

**Function:** converts the fermentation by-product hydrogen into methane.

**Health impact:** vital for nutrient and calorie absorption but can cause constipation, bloating and gas

+



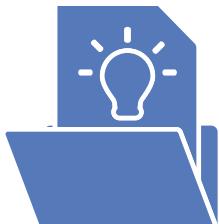
*Escherichia coli* is a Gram-negative, rod-shaped bacterium. It normally produces energy-rich molecules in aerobic respiration (i.e. with oxygen) but it can switch to fermentation if no oxygen is present.

**Healthy gut:** ubiquitous and commonly found in the gut

**Pathogenicity:** features such as fimbriae, capsule and toxins contribute to different symptoms

**Health impact:** pathogenic strains are associated with many conditions such as: urinary tract infection, neonatal meningitis or traveller's diarrhoea

**Transmission:** uncooked meat, contaminated water sources and others.



## Introducing microbes that can be found in the gut - part 2

*Campylobacter jejuni* is a Gram-negative and motile bacterium in the shape of a spiral-rod. This organism is a thermophile and can grow at 42°C.

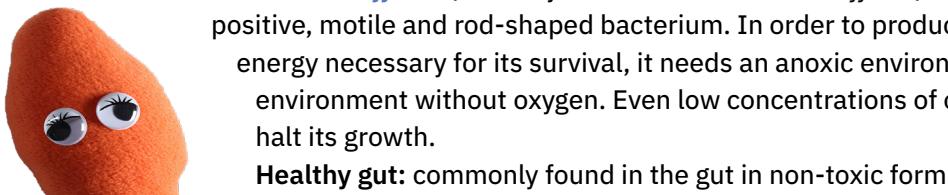


**Healthy gut:** absent in healthy individuals

**Pathogenicity:** colonizes intestinal cells and replicates intracellularly, causing cell damage.

**Transmission:** the main reservoir is undercooked poultry, contaminated water or unpasteurised milk.

*Clostridioides difficile* (formerly known as *Clostridium difficile*) is a Gram-positive, motile and rod-shaped bacterium. In order to produce the energy necessary for its survival, it needs an anoxic environment, i.e. an environment without oxygen. Even low concentrations of oxygen gas can halt its growth.



**Healthy gut:** commonly found in the gut in non-toxic form

**Pathogenicity:** can become an opportunistic pathogen when competition with other microbes is reduced for example following the use of antibiotics or as a result of diseases like inflammatory bowel disease.

**Health impact:** *C. difficile* produces a toxin (toxin A) that causes severe diarrhoea, cramps, nausea which can be potentially life-threatening

**Transmission:** A common hospital acquired infection; spores can survive harsh environments.

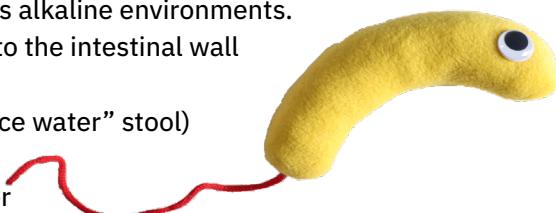
*Vibrio cholerae* is a Gram-negative, motile and curved-rod-shaped bacterium. This organism is an alkaliphile and prefers slightly basic conditions to thrive.

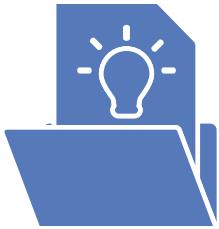
**Healthy gut:** absent in healthy individuals; prefers alkaline environments.

**Pathogenicity:** produces cholera toxin, attaches to the intestinal wall via fimbriae.

**Health impact:** results in secretory diarrhoea (“rice water” stool) and dangerously low blood pressure.

**Transmission:** transmitted in a faecal-oral manner due to poor sanitation





# Phylogenetic trees

Phylogenetics is the study of evolutionary relationships among taxonomic groups (e.g., organisms, species and populations) or other biological units (e.g., genes and proteins).

## Classical versus molecular phylogenetics

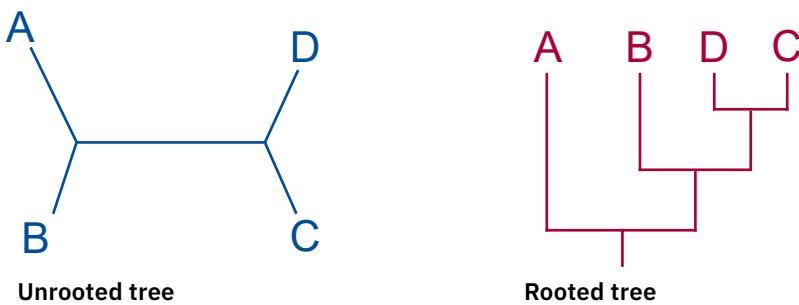
Phylogenetic reconstruction has been playing a key role in understanding evolutionary processes. Traditional classification methods have looked at the evolutionary relationships of classical biological units like phyla, species or populations, and were based on morphological observations and phenotypic characteristics. Advances in molecular biology within the past decades, however, have revolutionised phylogenetic studies. Especially techniques like DNA and whole genome sequencing, coupled with an increase in biological databases, have significantly extended the scope of phylogenetics and allowed scientists to build evolutionary trees on the basis of the molecular makeup of organisms. Through these developments, molecular data has now become the prime source for the construction of phylogenetic trees.

With the use of molecular data for the assessment of relatedness, identification of taxonomic common ancestors has become more accurate. This advancement not only offers vital clues on the evolutionary relationship of organisms but can also help identify new model organisms which are the closest resembling relatives to ancient common ancestors.

In phylogenetic trees, the degree of evolutionary relatedness is expressed in the positions of the individual units of interest (e.g., species, populations, genes) to each other. Units which are closely related are placed close together, while units which are more distantly related are positioned on different branches or clades in the tree.

## Tree basics

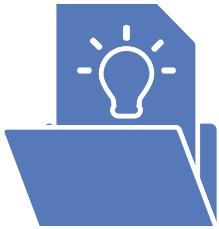
### Unrooted versus rooted phylogenetic trees



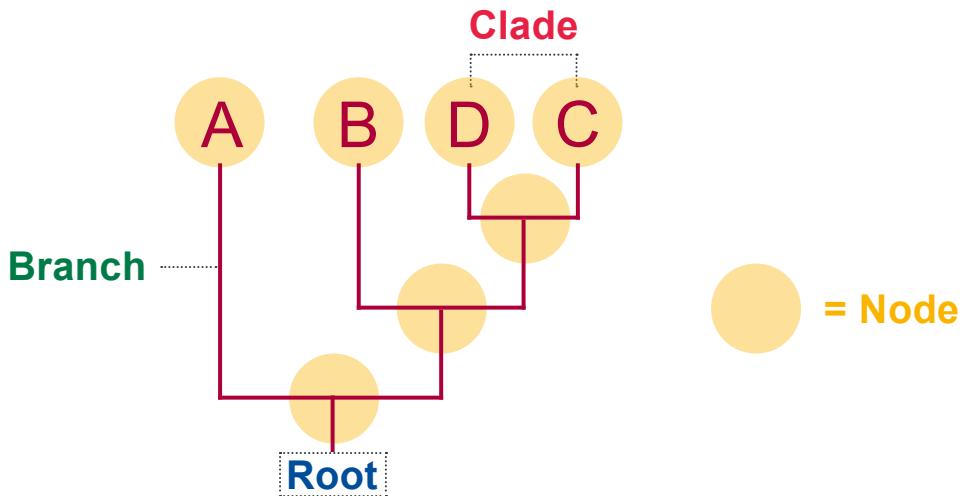
An unrooted tree does not provide any information about the common ancestor of the study group. An unrooted tree can be converted into a rooted tree – a tree which provides information about the common ancestor (root) of the study group. By comparing the trait of interest present in the study group (ingroup) with the trait present in a group which is only distantly related to the ingroup (outgroup), it can be determined whether the trait of interest is ancestral (present in common ancestor) or derived (not present in common ancestor).

#### Reference:

"TreeTOPS – a phylogeny icebreaker game"; <https://www.embl.org/ells/teachingbase/treetops/>.



# Phylogenetic terminology of rooted trees



## Branch

Connects the nodes of the tree and represents the proposed evolutionary path of the specific lineage.

## Root

Node which represents the common ancestor of all nodes in the tree.

## Node

Internal node: represents the hypothetical common ancestor of a clade

Terminal node (leaf): represents (usually present-day) taxon (population, species, gene, protein, etc.) which is being compared to other taxa in the tree.

## Clade

In a clade, all members share a common ancestor which is not the common ancestor of any other member of the tree.

## References:

"TreeTOPS – a phylogeny icebreaker game"; <https://www.embl.org/ells/teachingbase/treetops/>.

UC Museum of Paleontology; Understanding phylogenies; <https://evolution.berkeley.edu/evolution-101/the-history-of-life-looking-at-the-patterns/understanding-phylogenies/>

# Activity worksheet

## Introducing your microbiome

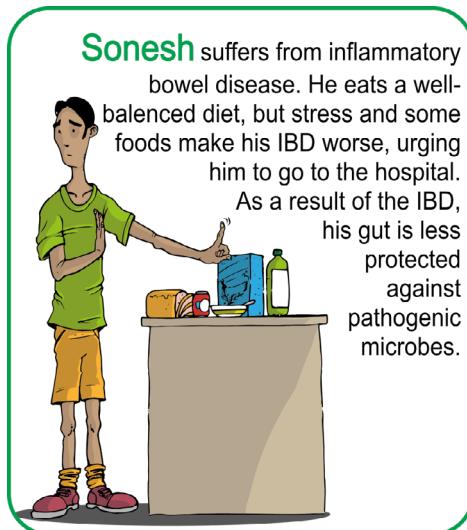
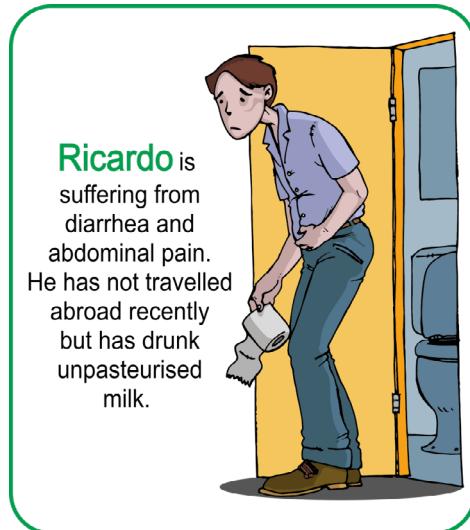
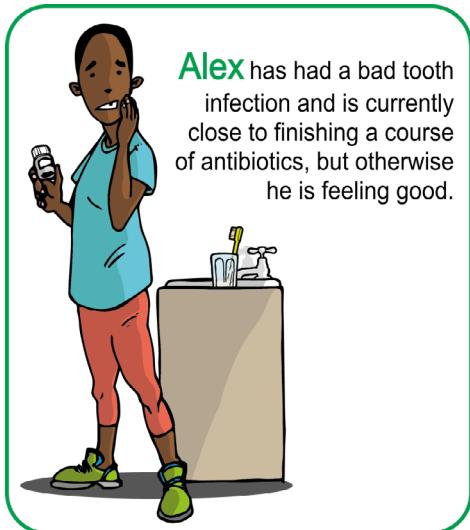
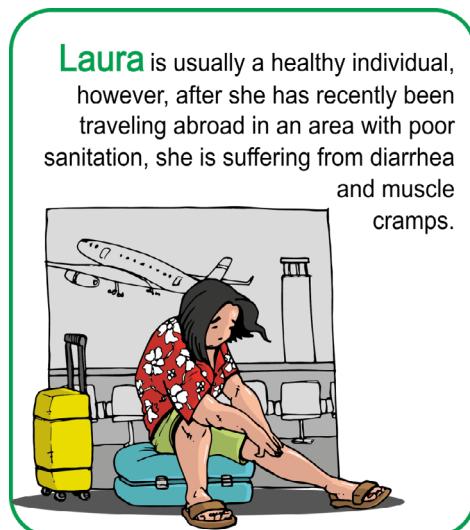
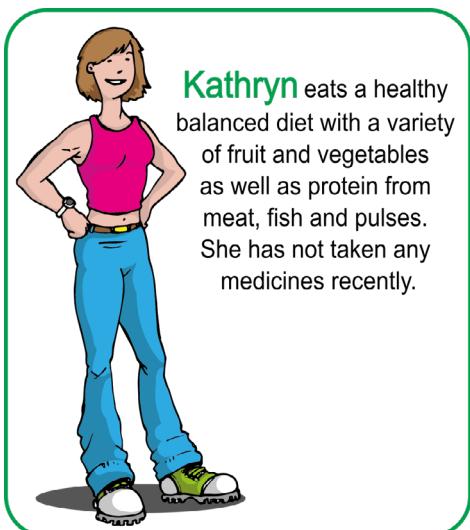
The educational resource package



## ACTIVITY 1 - MATCHING GUT MICROBIOMES

Read the profiles of Kathryn and her friends and the fact sheets about the different microbes. When looking at the “microbiome compositions”, note that the plus-symbols (+) indicate the relative abundance of the microbe in the whole microbiome.

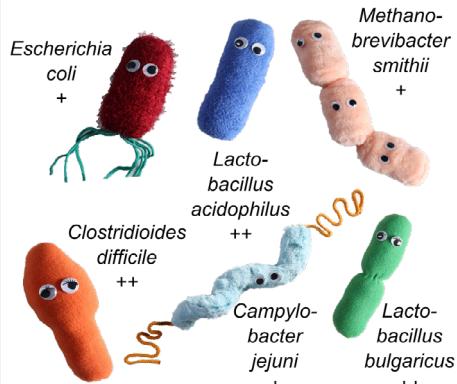
Using this information, try to match Kathryn and her friends to their respective gut microbiome compositions.



## ACTIVITY 1 CONTINUED

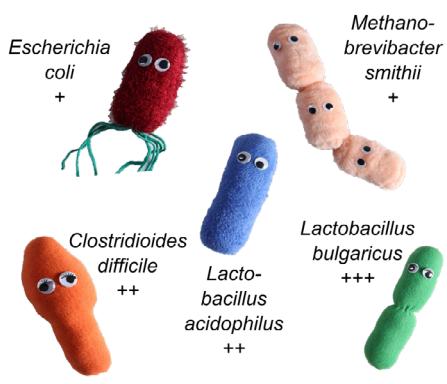
**Microbiome compositions:**

Microbiome composition 1



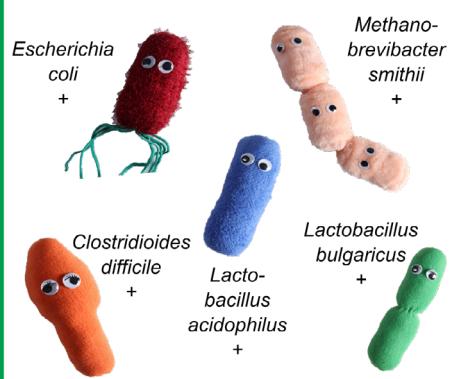
This microbiome belongs to:

Microbiome composition 2



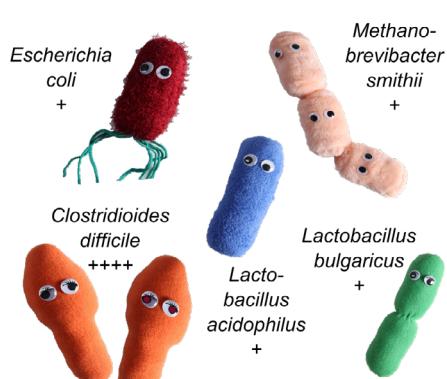
This microbiome belongs to:

Microbiome composition 3



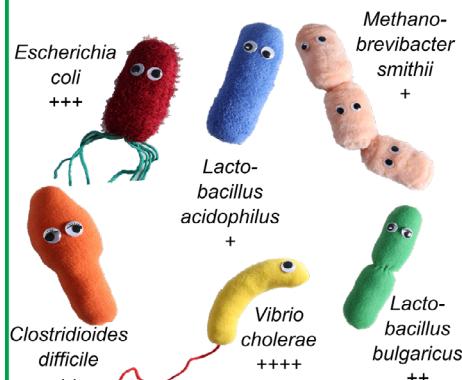
This microbiome belongs to:

Microbiome composition 4



This microbiome belongs to:

Microbiome composition 5



This microbiome belongs to:

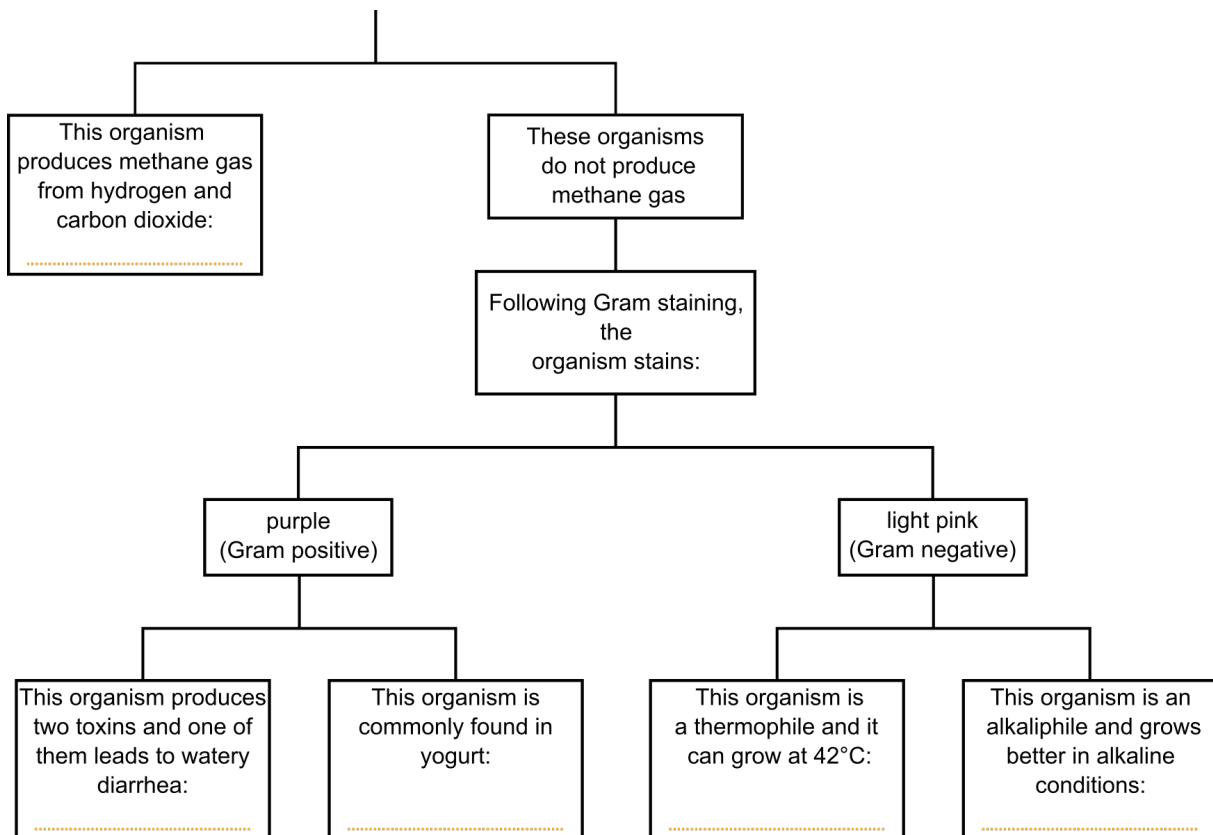
## ACTIVITY 2 - FLOWCHART DECISION TREE

**A**

The flowchart below is a representative dichotomous key that can be used to classify organisms according to certain characteristics.

**Look at the fact sheet containing information on the seven gut microbiome residents and fill in the blanks in the flowchart.**

Note that there are two microbes you will not be able to distinguish.



**B**

Scientists often use physiological and biochemical properties such as shape, response to Gram staining, optimal growth conditions and cellular respiration end products to distinguish between different microorganisms. However, such information sometimes falls short of differentiating between more closely related organisms.

**B a**

**Identify and note down the two organisms that you could not separate using the flowchart.**

**ACTIVITY 2 - CONTINUED****Bb**

Below, you will find a table comparing *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*.

Based on the table, identify one characteristic that can be used to distinguish these two species.

---

Characteristic	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus bulgaricus</i>
Gram staining	positive (purple)	positive (purple)
Shape	rod (bacillus)	rod (bacillus)
Energy production	lactic acid fermentation	lactic acid fermentation
Short section of the 16S rRNA genomic sequence	ACGGCTAACCGTGGAA	TGGGCTTAACCGAGGAA

## ACTIVITY 3 - HOW COMPUTERS DRAW A PHYLOGENETIC TREE

When computers draw a phylogenetic tree, they do not start with microbial characteristics like shape or response to Gram staining, but they draw them based on RNA or DNA sequences. Let's see how they do this:

### A finding sequence similarities

First, computers align the nucleotide sequences. This means that they look for similar nucleotide stretches across the sequences and arrange them accordingly.

Below, you will find four aligned sequences, each containing 10 nucleotides. For the nucleotides in 3rd and 10th position, similarities across sequences are already colour-shaded.

**Complete the table by colouring or highlighting nucleotides that are the same across two or more sequences in each of the 10 positions.**

	1	2	3	4	5	6	7	8	9	10
Sequence 1	A	G	A	C	C	T	A	C	C	C
Sequence 2	A	T	C	G	G	A	T	C	C	C
Sequence 3	A	G	C	G	G	A	T	C	C	C
Sequence 4	A	G	A	C	G	A	T	C	C	C

### B estimating relatedness by calculating distances

Next, using the alignment, computers can determine relatedness between the sequences –and thus the different species- since more closely related microbes will share more similar sequences. Relatedness can be quantified using various so-called distance metrics. Such distance metrics can be used to generate phylogenetic trees, which hypothesise how closely different organisms are related.

How do computers calculate the distances? One option is to determine the number of nucleotides that differ between two sequences at a given position, so-called mismatches. Dividing the mismatches by the total number of nucleotides in the alignment ( $n=10$ ) identifies the distance metric.

As an example, calculating the distance between Sequence 1 and 2 would look like this:

	1	2	3	4	5	6	7	8	9	10
Sequence 1	A	G	A	C	C	T	A	C	C	C
Sequence 2	A	T	C	G	G	A	T	C	C	C

Number of mismatches: 6

Total number of nucleotides in alignment: 10

Distance: 6/10

**ACTIVITY 3 - CONTINUED****B** a

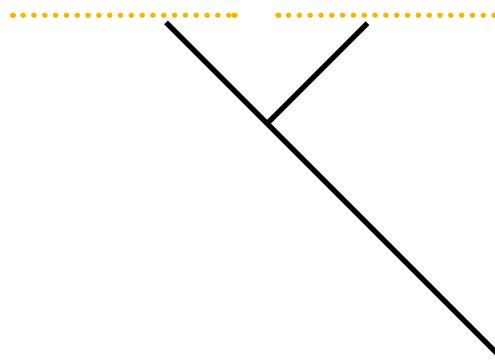
Following this example, fill out the boxes with the dashed lines in the table below with the calculated distance for the respective sequence pairs.

	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Sequence 1	$0/10 = 0$	$6/10$	$5/10$	.....
Sequence 2	-	$0/10 = 0$	.....	.....
Sequence 3	-	-	$0/10 = 0$	.....
Sequence 4	-	-	-	.....

Which two sequences have the shortest distance (i.e. the least mismatches) between each other? What does this tell you about their genetic relatedness?

The shortest distance is between Sequence ..... and Sequence ..... . The shortest distance infers the ..... genetic relatedness.

You can therefore connect these two sequences to form the first two branches of the future tree below. Note that the distances here are not to scale.



**ACTIVITY 3 - CONTINUED****Bb**

Now, a computer can re-calculate the distances.

To this end, it considers the first two sequences as one group and calculates their combined distance to the other sequences.

We will calculate the distance between Sequence 1 and [Sequence 2-Sequence 3] by taking the average of the distance between “Sequence 1 versus Sequence 2” and “Sequence 1 versus Sequence 3”. You have calculated these distances in exercise **Ba** of this activity.

**Following the example you find in the table, fill out the boxes with the dashed lines in the table below.**

	Sequence 1	[Sequence 2-Sequence 3]	Sequence 4
Sequence 1	$0/10 = 0$	$1/2(6/10+5/10) = 11/20$	.....
[Sequence 2-Sequence 3]	-	$1/2(0/10+0/10) = 0$	.....
Sequence 4	-	-	.....

**Bc**

Which sequences is the next closest to the sequence pair [Sequence 2-Sequence 3]?

The next closest sequence is Sequence ..... .

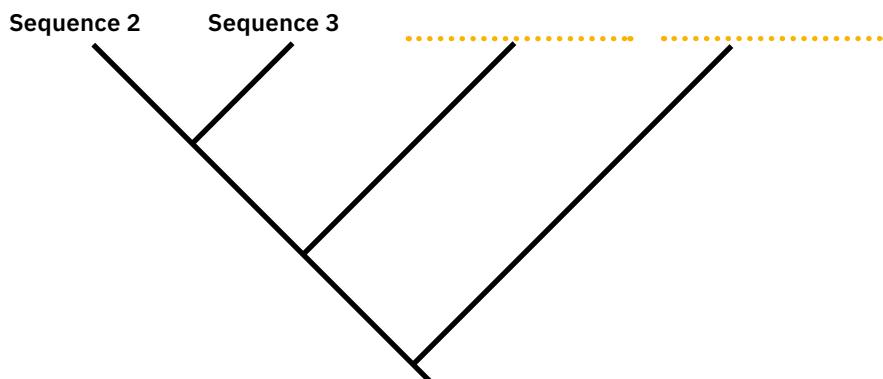
**Bd**

Which sequences is the farthest from every other sequence?

Sequence ..... is farthest from every other sequence.

**Be**

Construct the simplest tree that explains the alignment:

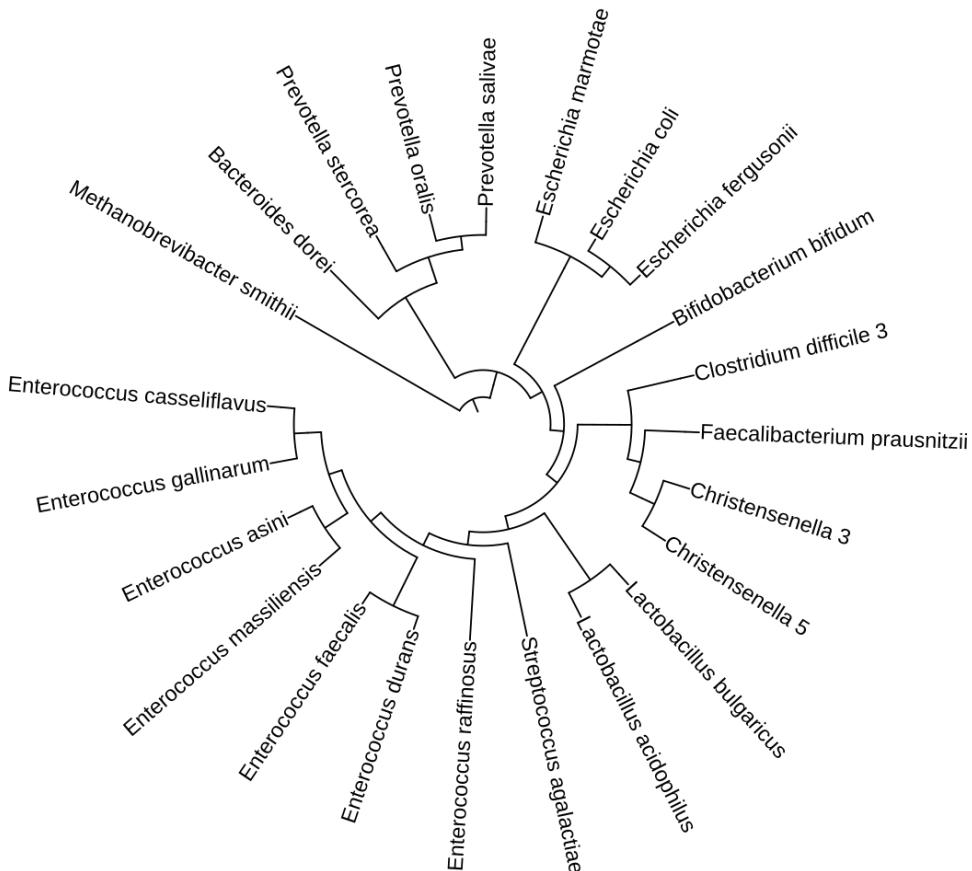


Reference:

Stanford at The Tech, Understanding Genetics; Evolution; <https://genetics.thetech.org/ask-a-geneticist/how-build-phylogenetic-tree>

## ACTIVITY 4 - PAPER-BASED ITOL ALTERNATIVE

The cladogram below shows the relationship between different microbes living in the human gut. The phylogenetic tree below was constructed using metagenomic sequencing data of entire bacterial genomes. The genetic material for this analysis stems directly from stool samples.



**Figure 1**

A

Based on the tree and the [flash card "Phylogenetic terminology of rooted trees"](#), identify the last common ancestor for all microorganisms.

B

The box below contains a group of microorganisms that are found in the human gut.

*Bacteroides dorei*, *Lactobacillus acidophilus*, *Escherichia coli*, *Methanobrevibacter smithii*, *Streptococcus agalactiae*, *Clostridioides difficile*

List them starting from the most closely related to the least closely related species to *Lactobacillus bulgaricus*.

**ACTIVITY 4 - CONTINUED****C**

In the tree, identify and circle the nodes that represent:

**C a**

The last common ancestor of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*.

**C b**

The last common ancestor of *Lactobacillus bulgaricus* and *Clostridioides difficile*.

**D**

The gut microbiome is composed of various microorganisms such as bacteria and archaea. Bacteria like *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* use carbohydrates in the gut for production of energy-rich molecules. On the other hand, some archaea such as *Methanobrevibacter smithii* can use bacterial waste products like carbon dioxide ( $\text{CO}_2$ ) and hydrogen ( $\text{H}_2$ ) to generate energy-rich molecules. The underlying chemical reaction produces methane gas ( $\text{CH}_4$ ).

Based on the short text above, identify one characteristic that supports the early branching of *M. smithii* from bacteria such as *L. bulgaricus* and *L. acidophilus*.

---

## ACTIVITY 4 - CONTINUED

E

Take a look at the table below showing a comparison of gene sequences from different microorganisms. The table shows short DNA sequences taken from a ribosomal RNA gene called the 16S ribosomal RNA (rRNA). The 16S rRNA gene is present in all prokaryotes and comparing the sequence of this gene across different prokaryotes can help us infer evolutionary relatedness among organisms. The sequences shown are representative of the overall 16S rRNA gene similarity between the species in the table below.

**Based on the sequence similarities and the organisms' overall position in the cladogram provided in Figure 1, match the rest of the species in the box below with the sequences provided in the table.**

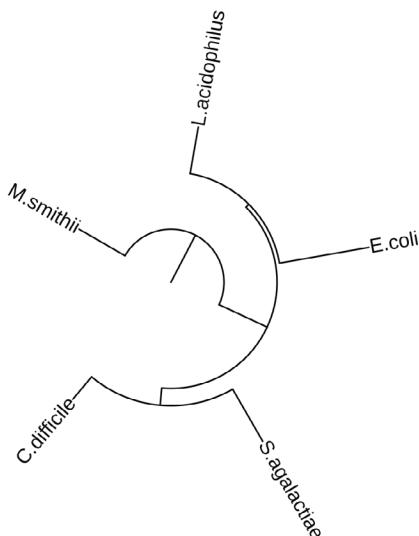
Highlight matching nucleotides across the sequences to make identifying the similarities easier.

*Lactobacillus acidophilus, Streptococcus agalactiae, Clostridioides difficile, Escherichia coli, Methanobrevibacter smithii*

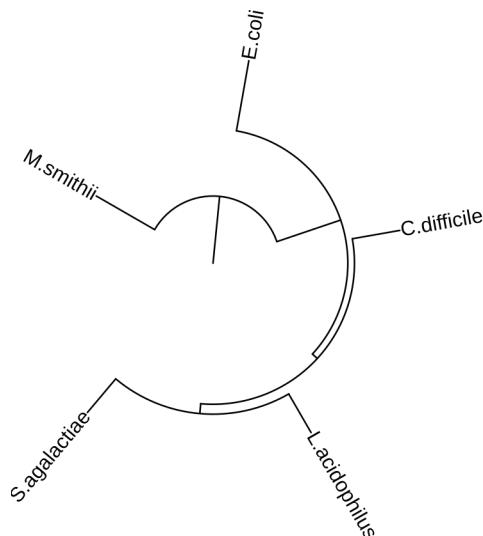
		Sequence																				Microorganism	
G	T	T	A	G	A	G	T	A	C	T	C	C	A	G	G	T	A	G	G	T	G	A	A
G	G	G	G	G	T	A	G	A	A	T	T	C	C	A	G	G	T	G	T	A	G	C	G
G	A	G	A	G	T	G	G	A	A	T	T	C	C	T	A	G	T	G	A	G	C	G	T
G	A	G	A	G	T	G	G	A	A	T	T	C	C	A	T	G	T	G	A	A	G	G	T
G	A	G	A	G	T	G	G	A	A	T	T	C	C	A	T	G	T	G	A	A	G	G	T
G	A	G	A	G	T	G	G	A	A	T	T	C	C	A	T	G	T	G	A	A	G	G	T

**ACTIVITY 4 - CONTINUED****F**

These are two cladograms generated using the nucleotide sequence information from the 16S rRNA genes of five different microorganisms:



*Phylogenetic tree generated using approximately 140 nucleotides of the 16S rRNA gene sequence*



*Phylogenetic tree generated using all approximately 1500 nucleotides of the 16S rRNA gene sequence*

**F a**

In both trees, identify the closest relative of *Lactobacillus acidophilus*.

.....  
.....

**F b**

Compare the two cladograms above with the evolutionary tree in **Figure 1**.

**Identify the cladogram that represents the kinship among the five microorganisms more accurately.**

.....

**F c**

Hypothesise why your cladogram of choice might be representing the kinship among the five microorganisms more accurately than the other one.

.....  
.....  
.....

## ACTIVITY 5 - WORKING WITH PHYLOGENETIC TREES IN iTOL

In this activity, you will visualise the gut microbiomes of the individuals you met in Activity 1 to compare them with each other.

### A

Before you start using the programme, gain a basic overview of the iTOL user interface, its basic functions and how to annotate trees by watching the video tutorial and reading the [iTOL basics guide](#).

#### A a

Following these instructions, go to the iTOL webpage (<https://itol.embl.de/>) and, in case you have an account, log in.

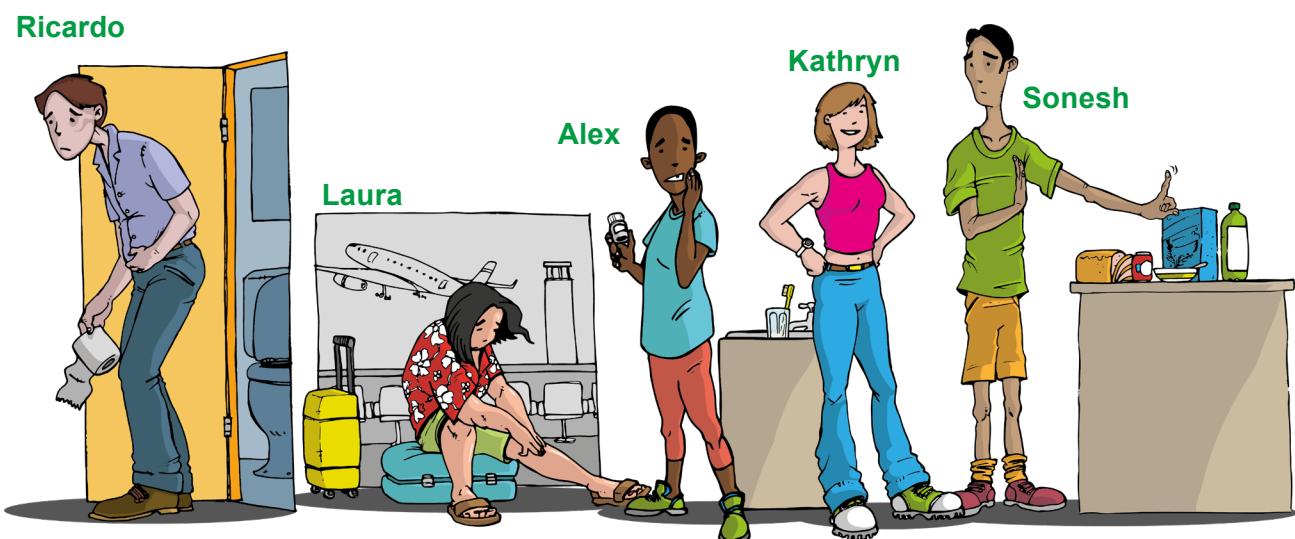
Upload the provided data set trees for each person (Alex.nwk; Kathryn.nwk; Laura.nwk; Ricardo.nwk and Sonesh.nwk).

Annotate the trees using the `itol_status.txt` and the `itol_taxa.txt` files.

#### A b

##### Explore the trees!

Switch between circular and rectangular representation, root the trees around the archaeon *Methanobrevibacter smithii* or find microorganisms of interest.



**ACTIVITY 5 - CONTINUED****B**

Based on your observations, answer the following questions.

**B a**

**Identify three microorganisms that are consistently present across the gut microbiome profiles. Why do you think this is?**

.....  
.....  
.....  
.....  
.....

**B b**

Sonesh is suffering from a *C. difficile* infection.

**How many *C. difficile* strains are present in his microbiome?**

There are ..... strains present.

**B c**

**Comparing Sonesh's tree with the others, which strain is most likely causing his problems?**

.....

**B d**

**Which organism is present only in Laura's gut microbiome and could indicate the cause of her illness?**

.....

**B e**

Ricardo has similar symptoms to Laura.

**Comparing their gut microbiomes are they suffering from the same disease or is there a different cause?**

.....

**B f**

Compare Alex's gut microbiome composition to Kathryn's.

**Has the microbe composition been affected by taking antibiotics?**

.....  
.....  
.....

# iTOL basics guide

## Introducing your microbiome

The educational resource package



# iTOL basics guide

## How to work with iTOL - Step by step

(with an iTOL account)

### 1. Download the data files for this activity.

These files include 5 files ending in .nwk and two text files (itol\_taxa.txt and itol\_status.txt). The files ending in .nwk are in the Newick format, which is a textual format for representing trees. Other formats supported by iTOL are Nexus, PhyloXML and Jplace. You can open the newick files using a text editor to visualize the contents. A simple example of a newick file will look like this:

```
(A:0.1, (B:0.1, C:0.1) 90:0.1) 98:0.3);
```

Here, A, B, and C are the leaf names, 0.1 and 0.3 are the branch lengths, and 90, 98 are the bootstrap values. A high bootstrap value, which ranges from 0-100, indicates that there is high confidence in the tree topology.

### 2. Login to iTOL ([https://itol.embl.deitol\\_account.cgi](https://itol.embl.deitol_account.cgi)).

Once you have successfully logged in, a “My Trees” tab along the top toolbar will appear. Click on this tab.

### 3. Click on the functions menu **1** and then click on “Create a project” **2**. For the Project name **3** enter your first and last name so your work will be identifiable

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
16s_rRNA_whole_gene	no description	5	no datasets	no views	Ψ	2021-11-18	2021-11-18 07:45	2021-11-18 13:41
16S_around_v1&2(approx.140 nucleotides)	no description	5	no datasets	no views	Ψ	2021-11-18		2021-11-18 07:59
Kathryn.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:50
Laura.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-23 18:09
Ricardo.nwk	no description	41	no datasets	no views	Ψ	2021-11-19		
Sonesh.nwk	no description	35	no datasets	no views	Ψ	2021-11-19		2021-11-19 13:27
Alex.nwk	no description	33	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:17

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
16s_rRNA_whole_gene	no description	5	no datasets	no views	Ψ	2021-11-18	2021-11-18 07:45	2021-11-18 13:41
16S_around_v1&2(approx.140 nucleotides)	no description	5	no datasets	no views	Ψ	2021-11-18		2021-11-18 07:59
Kathryn.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:50
Laura.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-23 18:09
Ricardo.nwk	no description	41	no datasets	no views	Ψ	2021-11-19		
Sonesh.nwk	no description	35	no datasets	no views	Ψ	2021-11-19		2021-11-19 13:27
Alex.nwk	no description	33	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:17

4. Once you have created your project, a new project box should appear with your name.

Click on the “Upload tree files” button **4** and select all the tree files **5** (ending in .nwk) you downloaded from eCampus and press upload. The trees should now appear in your workspace **6**.

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
16S_rRNA_whole_gene	no description	5	no datasets	no views	Ψ	2021-11-18	2021-11-18 07:45	2021-11-18 13:41
16S_around_v1&2(approx.140 nucleotides)	no description	5	no datasets	no views	Ψ	2021-11-18		2021-11-18 07:59
Kathryn.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:50
Laura.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-23 18:09
Ricardo.nwk	no description	41	no datasets	no views	Ψ	2021-11-19		
Sonesh.nwk	no description	35	no datasets	no views	Ψ	2021-11-19		2021-11-19 13:27
Alex.nwk	no description	33	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:17

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
There are no trees in this project								

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
16S_rRNA_whole_gene	no description	5	no datasets	no views	Ψ	2021-11-18	2021-11-18 07:45	2021-11-18 13:41
16S_around_v1&2(approx.140 nucleotides)	no description	5	no datasets	no views	Ψ	2021-11-18		2021-11-18 07:59
Kathryn.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:50
Laura.nwk	no description	45	no datasets	no views	Ψ	2021-11-19		2021-11-23 18:09
Ricardo.nwk	no description	41	no datasets	no views	Ψ	2021-11-19		
Sonesh.nwk	no description	35	no datasets	no views	Ψ	2021-11-19		2021-11-19 13:27
Alex.nwk	no description	33	no datasets	no views	Ψ	2021-11-19		2021-11-22 16:17

5. Click on a file **[7]** to view the associated tree **[8]**.

Familiarise yourself with the panels/display. The main part of the display is the tree. Try moving around by clicking and dragging using your mouse. You can zoom in and out using the and buttons. will fit the tree to screen.

The screenshot shows the ITOL interface with the following components:

- Header:** ITOL Interactive Tree Of Life, Tree of Life, My Trees, Data sharing, Help.
- User Profiles:**
  - Efrain Cufa:** No description. Shows a table of 7 trees:
 

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
16S_rRNA_whole_gene	no description	5	no datasets	no views	ψ	2021-11-18	2021-11-18 07:45	2021-11-18 13:41
16S_around_y1&2(approx.140 nucleotides)	no description	5	no datasets	no views	ψ	2021-11-18		2021-11-18 07:59
Kathryn.nwk	no description	45	no datasets	no views	ψ	2021-11-19		2021-11-22 16:50
Laura.nwk	no description	45	no datasets	no views	ψ	2021-11-19		2021-11-23 18:09
Ricardo.nwk	no description	41	no datasets	no views	ψ	2021-11-19		
Sonesh.nwk	no description	35	no datasets	no views	ψ	2021-11-19		2021-11-19 13:27
Alex.nwk	no description	33	no datasets	no views	ψ	2021-11-19		2021-11-22 16:17
  - Joe Smith:** No description. Shows a table of 5 trees:
 

Tree	Description	Leaves	Datasets	Views	Features	Inserted	Updated	Accessed
Kathryn.nwk	no description	45	no datasets	no views	ψ	2021-11-24		
Laura.nwk	[7] description	45	no datasets	no views	ψ	2021-11-24		
Ricardo.nwk	no description	41	no datasets	no views	ψ	2021-11-24		
Sonesh.nwk	no description	35	no datasets	no views	ψ	2021-11-24		
Alex.nwk	no description	33	no datasets	no views	ψ	2021-11-24		
- Tree Display:** A phylogenetic tree showing relationships between various bacterial species. The tree is rooted at the bottom and branches upwards. The main display area is labeled "Main tree display".
- Zoom controls:** Located on the left side of the tree display, these controls allow for zooming in and out.
- Main control panel:** Located on the right side of the tree display, this panel contains various options for tree visualization and editing, such as "Control panel", "Basic", "Advanced", "Datasets", and "Export" tabs, and settings for "Mode", "Labels", "Branch options", and "Tree views".

## 6. The tree is currently unrooted.

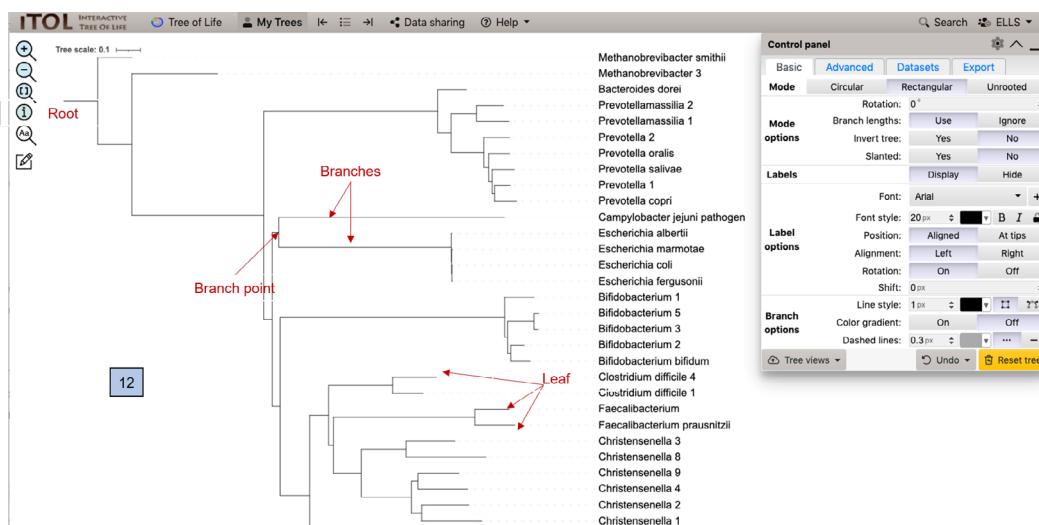
To root the tree, we need to choose an outgroup, a species that is most distantly related to the organisms in the tree.

To root the tree, right-click on the outgroup branch **9** (in this case, it is *Methanobrevibacter smithii*) and click on “Tree structure” **10**. Then click on “Re-root the tree here” **11**.

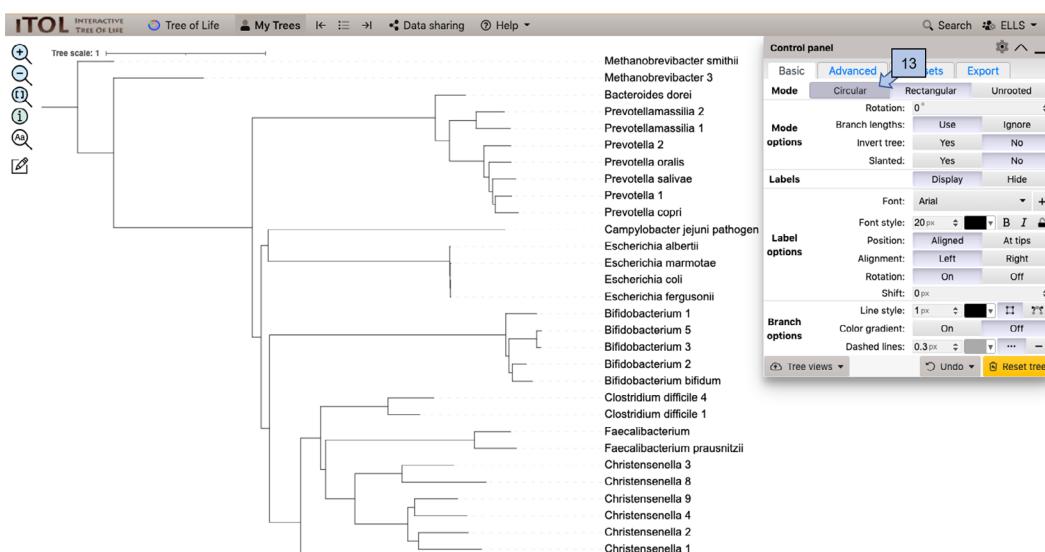
The screenshot shows a phylogenetic tree with various bacterial genera and species names listed on the right. A context menu is open at a node labeled '9'. The menu has several options: 'Leaf functions', 'Editing', and 'Tree structure'. The 'Tree structure' option is highlighted with a blue box and a numbered '10' below it. To its right, another option 'Re-root the tree here' is also highlighted with a blue box and a numbered '11' below it.

This screenshot shows the same phylogenetic tree as the previous one, but it has been re-rooted. The tree is now rooted at the node where the 'Re-root the tree here' option was selected. The context menu is still open at the original node 9, but the 'Tree structure' option is no longer highlighted.

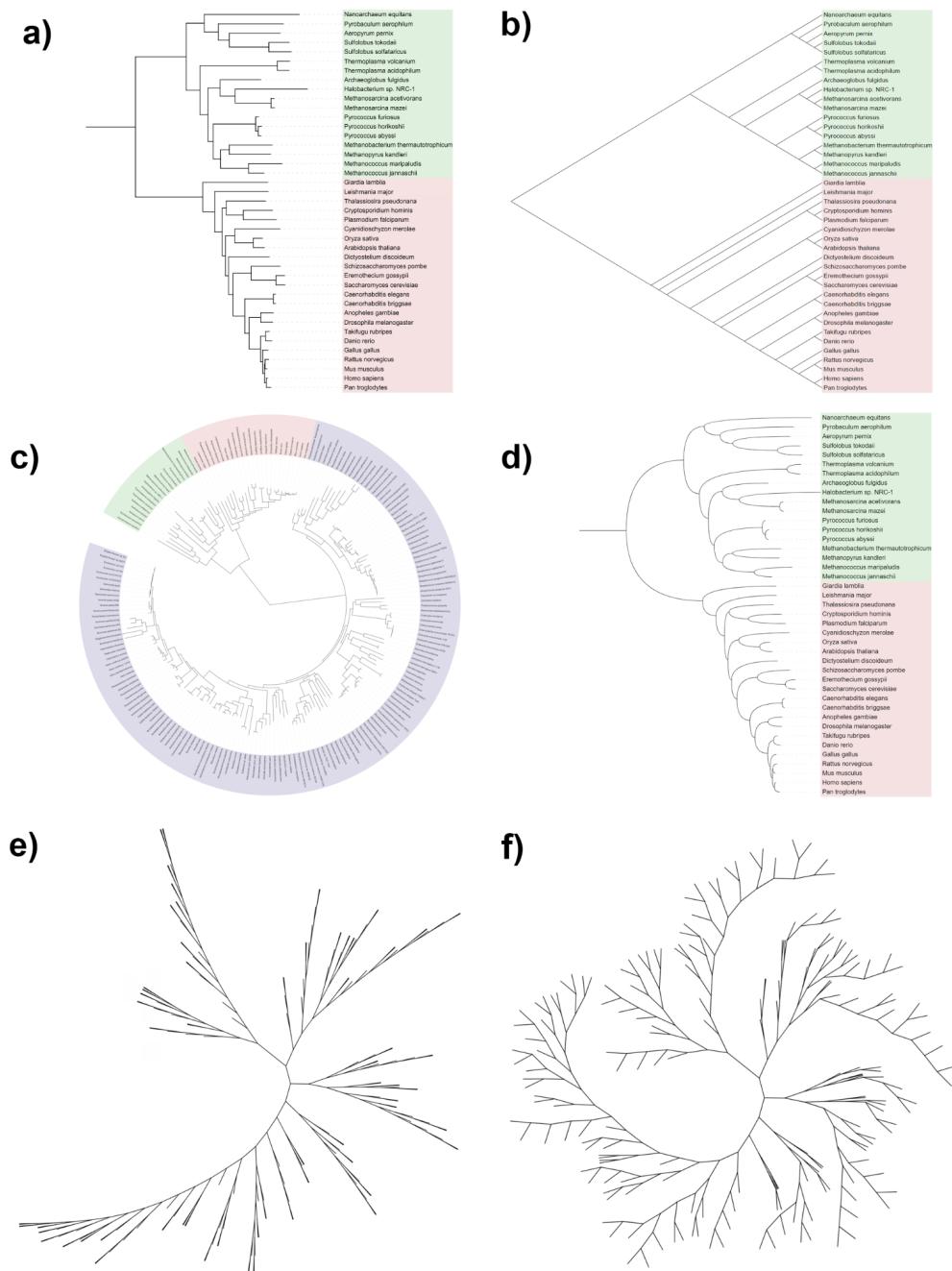
6. As previously discussed, a phylogenetic tree [12] is a diagram that allows us to visualize our hypotheses about the relationship between different organisms. The evolutionary relationship between different organisms can be inferred by their branching order on a tree. If they branched off more recently, they are more related. Branch points or internal nodes each represent a specific divergence of organisms.
- The tree allows us to visualize the diversity of species and strains that reside in the different gut microbiome samples.



7. Look at the main control panel. There will be different panels such as the “Basic”, “Advanced”, “Datasets”, and “Export”. These panels contain options that can help you better visualize your tree. To better view all of the tree leafs, change the tree “Mode” to circular view [13].

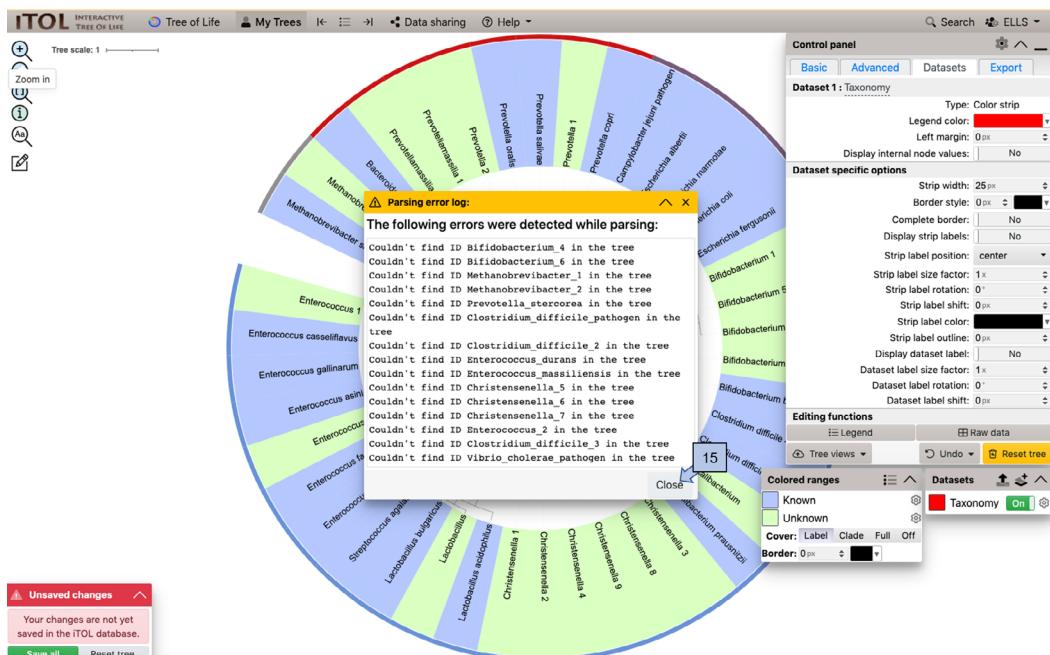
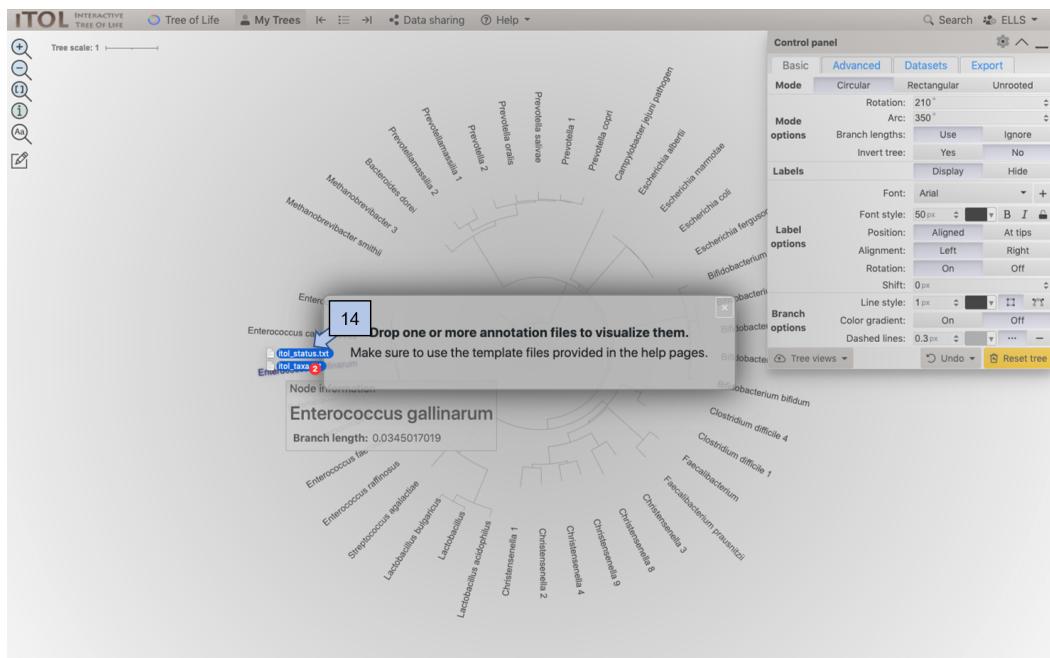


Other possible options can be achieved using “Mode”; “Mode options” are: a) Rectangular, b) Slanted (triangular), c) Circular d) Curved branches (in rectangular or circular modes), e) Unrooted (radial), f) equal-angle Unrooted (radial), equal-daylight (shown below). You can refer back to the tutorial videos ([https://itol.embl.de/video\\_tutorial.cgi](https://itol.embl.de/video_tutorial.cgi)) for guidance on how to explore the tree or change the tree display mode.



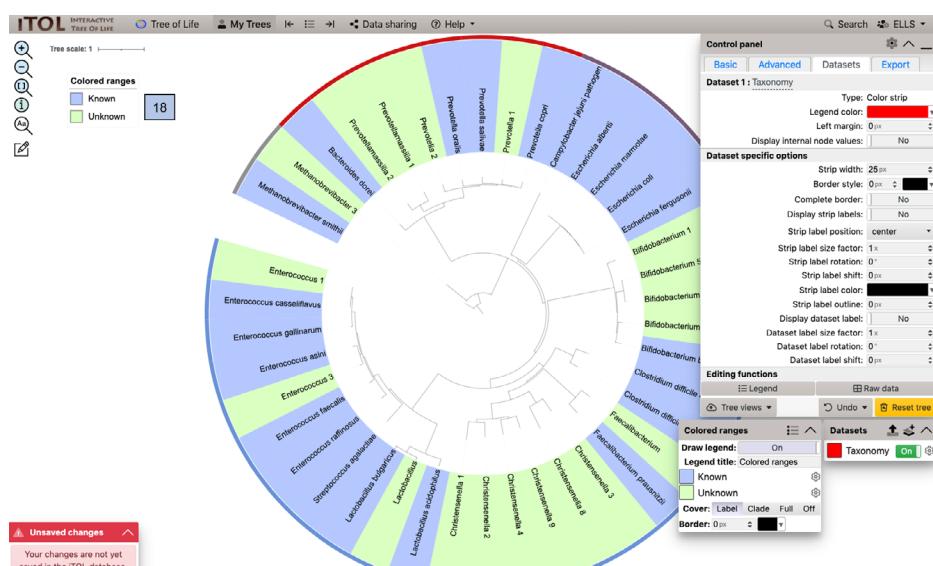
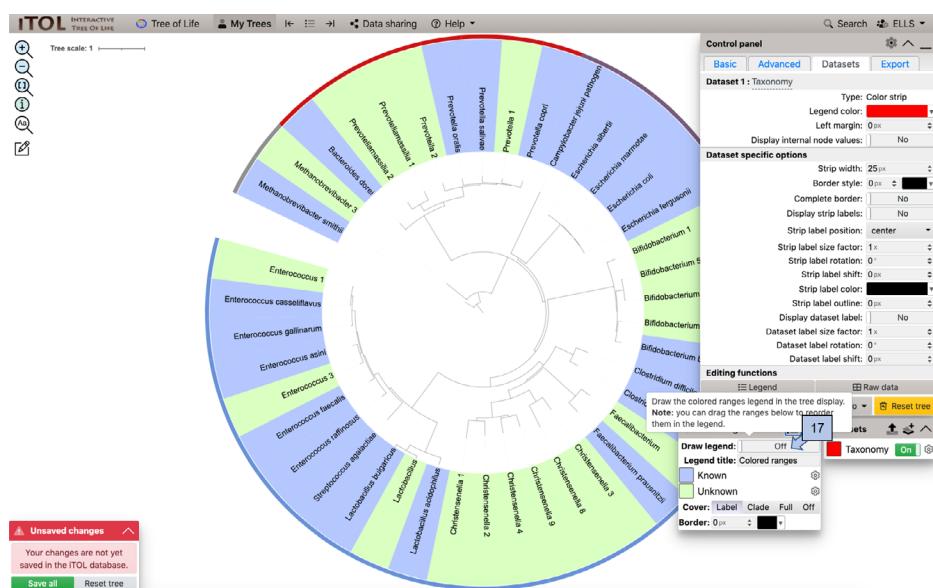
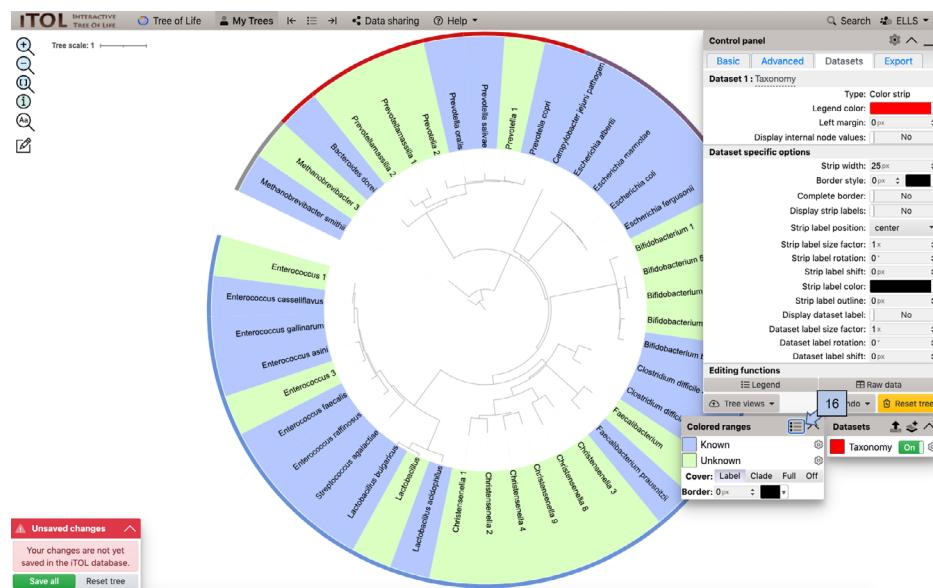
## 8. We can annotate our trees.

To add colour labels, click and drag **14** the `itol_taxa.txt` and `itol_status.txt` files from their downloaded location to the tree window (you can select and drag both files at once). For some trees you will get the following error message pop-up. Click “OK” **15** to dismiss the message, this is due to the nature of the file set-up.



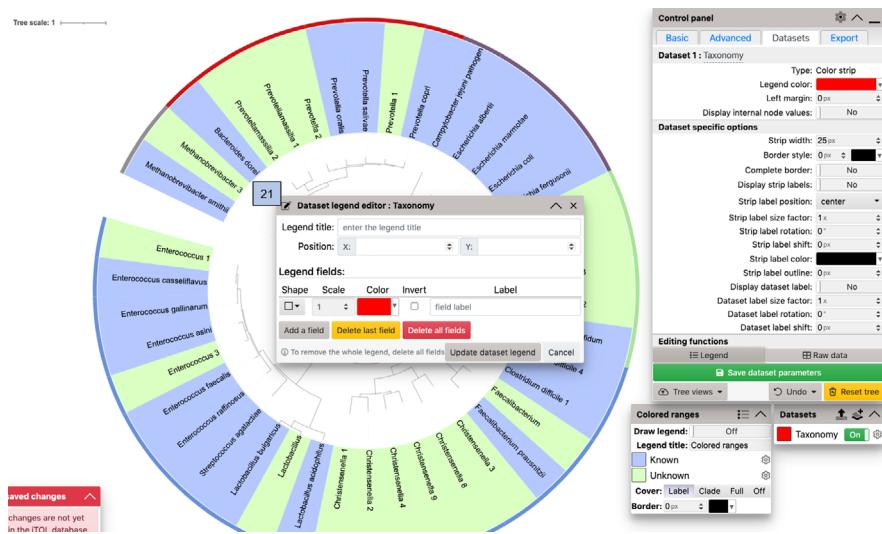
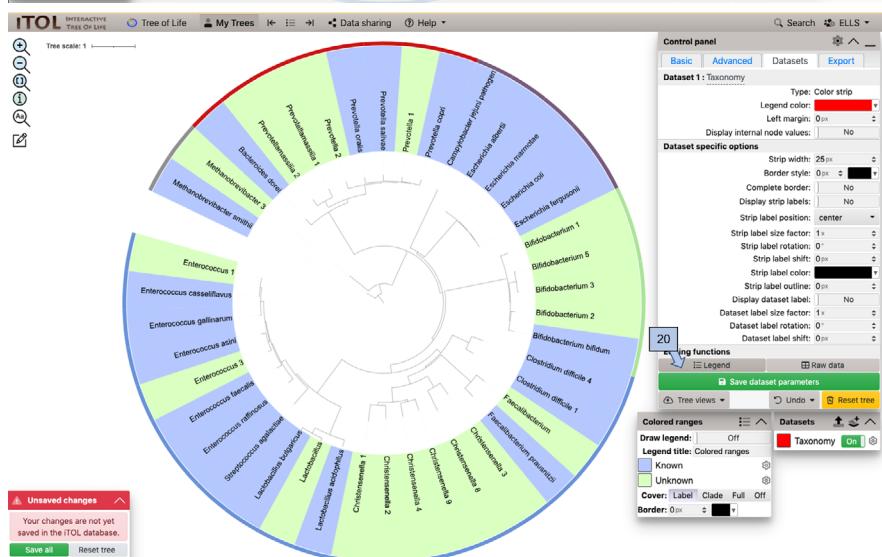
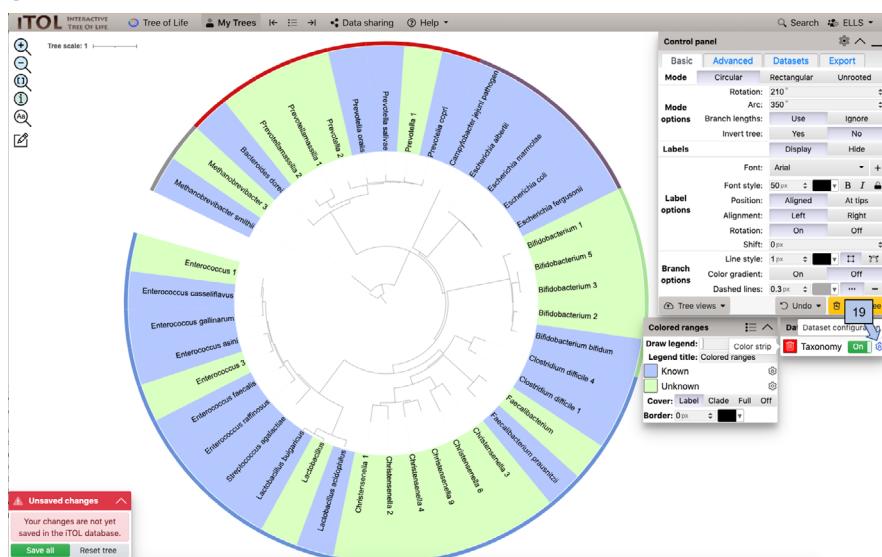
9. By default the legend will not be added.

To add the legend, click on “Colored ranges legend control” 16 and set “Draw legend” 17 from “Off” to “On”. The legend should now appear on the left 18.



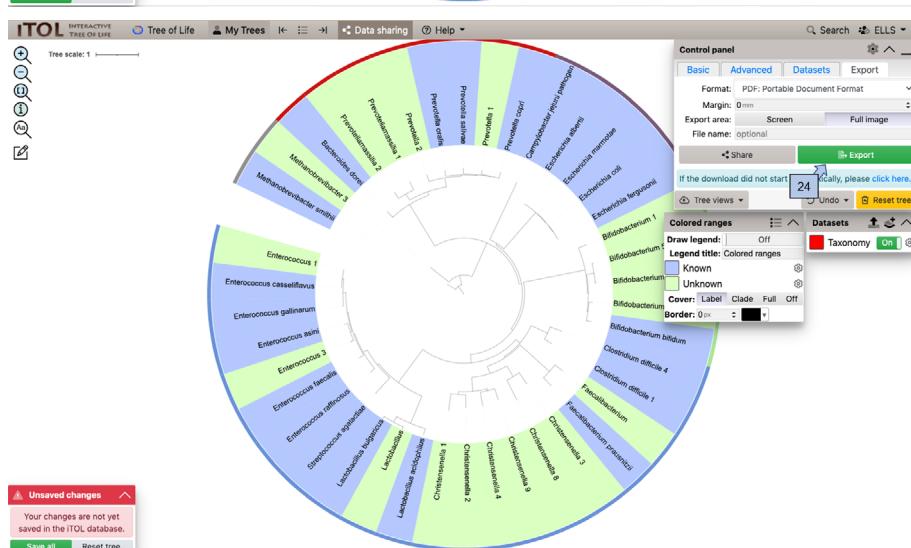
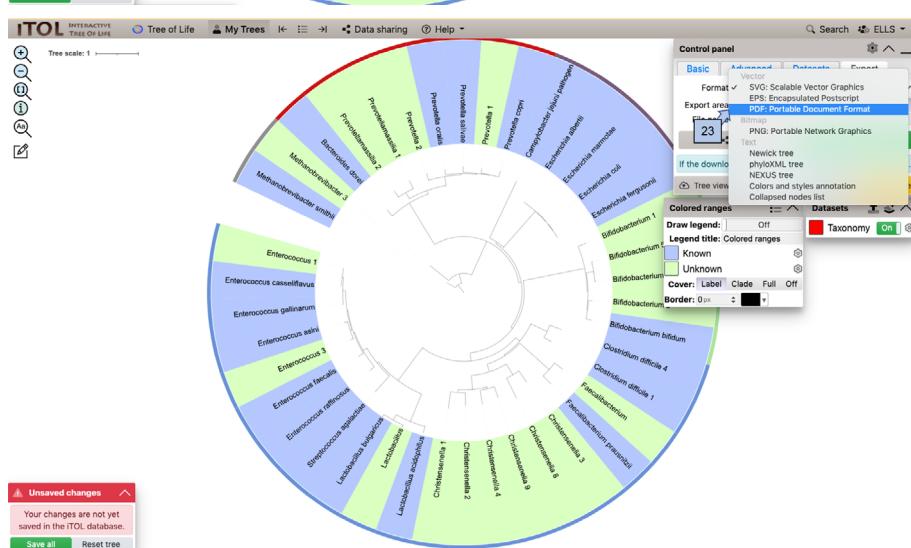
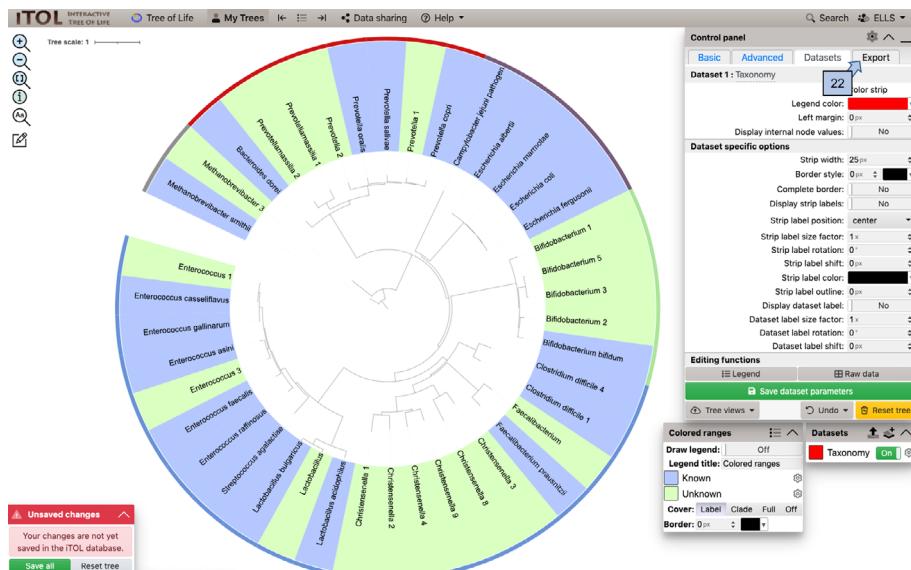
10. To make visualisation and exploration of the tree easier, you can label the datasets within your tree and create a Legend key.

Click the gear icon **19** in the “Datasets” panel. Select the “Datasets” tab in the “Controls” window. Next, click the “Legend” **20** button in the “Editing functions” section. This opens a pop-up **21** where you can label the taxonomy datasets within the tree and change the label colours. The “Legend title” will be Taxonomy, you can then add legend fields by adding text to the “Label” field, i.e. Archaea and change the colour of each set.



## 11. Play around with the layout and colour ranges to adapt the visualisation of your trees.

When you are happy with them you can export your work as a PDF file. To do this, click the “Export” tab **22** in the “Controls” window menu, select PDF **23** from the “Format” drop-down menu and click the “Export” button **24**. The file will automatically download. Please note that the tree files for each individual show a representative set of ~50 gut microbiota, in reality there are closer to ~200.



# Annex

## Supplementary resources:

The article “How to Read a Phylogenetic Tree” introduces phylogenetic trees and explains how to read them step-by-step.	<a href="https://evolution-outreach.biomedcentral.com/articles/10.1007/s12052-010-0273-6">https://evolution-outreach.biomedcentral.com/articles/10.1007/s12052-010-0273-6</a>
The video “How do you read Evolutionary Trees?” describes the first time an Evolutionary Tree was used in a criminal court in America, and explains how to interpret the relatedness of organisms on an Evolutionary Tree.	<a href="https://www.youtube.com/watch?v=Xd6Tc7tGOec">https://www.youtube.com/watch?v=Xd6Tc7tGOec</a>
The video “There are a million wrong ways to read a phylogenetic tree” gives an overview of the many ways a phylogenetic tree can be interpreted incorrectly and walks you through the only right way to read them.	<a href="https://www.youtube.com/watch?v=SqmbIucbOzk">https://www.youtube.com/watch?v=SqmbIucbOzk</a>
The video by “Amoeba Sisters” explores classification in biology as well as taxonomy hierarchy. It also discusses the importance of scientific names and why classification can have major changes due to DNA and cell structure evidence.	<a href="https://www.youtube.com/watch?v=DVouQRAKxYo&amp;list=PLwLOMyd7Dk1FuT0I6icE7octRIgJjqMBhS&amp;index=1">https://www.youtube.com/watch?v=DVouQRAKxYo&amp;list=PLwLOMyd7Dk1FuT0I6icE7octRIgJjqMBhS&amp;index=1</a>
The interactive resource “Creating Phylogenetic Trees from DNA Sequences” explores how DNA sequences can be used to understand evolutionary relationships among organisms and how to represent them as phylogenetic trees.	<a href="https://www.biointeractive.org/classroom-resources/creating-phylogenetic-trees-dna-sequences">https://www.biointeractive.org/classroom-resources/creating-phylogenetic-trees-dna-sequences</a>
This short article describes step-by-step the process of building a phylogenetic tree with a simple example.	<a href="https://evolution.berkeley.edu/evolibrary/article/phylogenetics_07">https://evolution.berkeley.edu/evolibrary/article/phylogenetics_07</a>
This article explains step by step how scientists construct phylogenetic trees and how they understand the degree of relatedness between living organisms.	<a href="https://genetics.thetech.org/ask-a-geneticist/how-build-phylogenetic-tree">https://genetics.thetech.org/ask-a-geneticist/how-build-phylogenetic-tree</a>
The icebreaker game “TreeTOPS” aims to introduce the players to phylogenetics. The players work in a group and build their own phylogenetic tree to illustrate the evolutionary branching process of an imaginary taxonomic group or biological unit. The activity is available in English, German, Czech and Greek.	<a href="https://www.embl.org/ells/teachingbase/treetops/">https://www.embl.org/ells/teachingbase/treetops/</a>

An introductory article on the Interactive Tree Of Life (iTOL).	<a href="https://itol.embl.de/help/17050570.pdf">https://itol.embl.de/help/17050570.pdf</a>
This article presents the latest developments around the iTOL ( <a href="https://itol.embl.de">https://itol.embl.de</a> ) tool, which allows for the display, manipulation and annotation of phylogenetic and other trees.	<a href="https://academic.oup.com/nar/article/47/W1/W256/5424068">https://academic.oup.com/nar/article/47/W1/W256/5424068</a>
A series of iTOL introductory video tutorials.	<a href="https://itol.embl.de/videoTutorial.cgi">https://itol.embl.de/videoTutorial.cgi</a>
iTOL help page for a more advanced iTOL guide.	<a href="https://itol.embl.de/help.cgi">https://itol.embl.de/help.cgi</a>

**NOTES**

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**Version 1: November 2021**