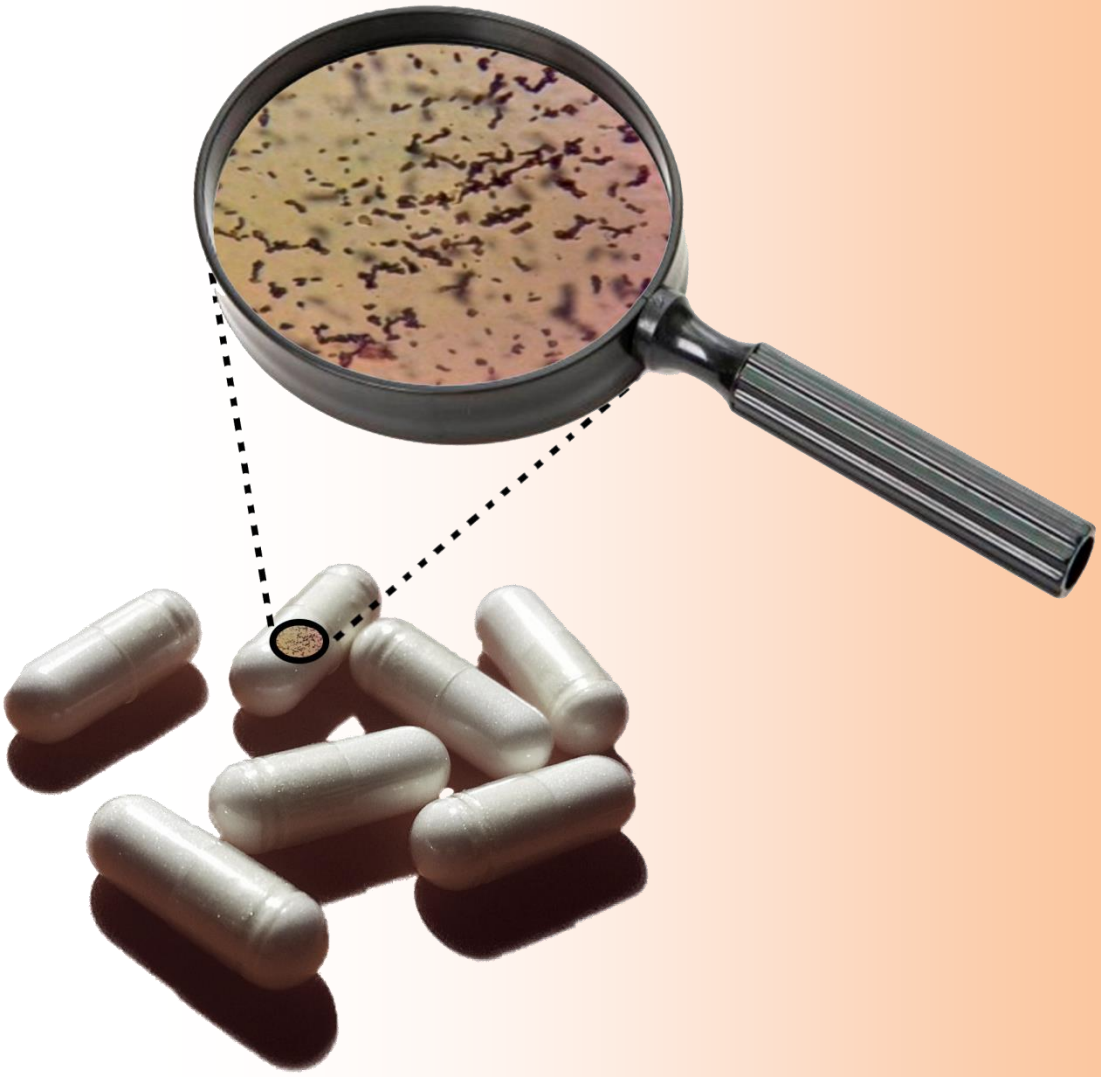
LIFE INSIDE US

A study about the efficacy of probiotics



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18th/10/2017

“Bacteria keep us from heaven and put us there.”

Martin H. Fischer

Acknowledgements

I have worked with great effort so as to put the best of me in this research. However, it would have been harder without the help and support of my tutor, family and friends.

First of all, I would like to thank Eva Méndez who supervised the redaction of the present work.

I would not forget to mention my friends who encouraged me even when they could not manage to understand what “Plankflat” and “microbiotics” were.

Furthermore, I would like to express my gratitude to my tutor, Fina Mengíbar, who helped me in everything she could and more. Thanks for teaching me many things that I will always be grateful for.

Finally, I would like to thank my family for their unconditional support. I am infinitely thankful for you being always on my side.

Clarifications

The words marked with an asterisk are included in the glossary with a brief explanation of the term. For instance, catalase-negative* and by clicking to the word, the reader will be redirected to the glossary.

Abbreviations

- **ADH**: Alcohol dehydrogenase
- **BF**: Breast-fed
- **Bif**: Bifidobacteria
- **CYP2E1**: Cytochrome P450-depentdent ethanol-oxidizing system
- **FF**: Formula-fed
- **GIT**: Gastrointestinal tract
- **IECs**: Intestinal epithelial cells
- **L**: Lactobacillus
- **LAB**: Lactic acid-producing bacteria

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Abstract

The present work analyses the efficacy of probiotics and as a consequence, the health benefits of their intake for the microbiota.

Quite recently, considerable attention has been paid to the effectiveness of the referred bacteria and many theories have been developed around this topic. Owing to that, the factors which may be considered inhibitory for the probiotic action will be critically examined.

Furthermore, these bacteria take an essential role in the microbiota preservation which at its time, can be leveraged to confer many health advantages that will be described as well as the relation between the afore-said bacteria and the microbiota. Nevertheless, the benefits of the intake of probiotics could not be studied in a practical way due to the difficulties in obtaining representative microbiota samples.

After having realized several laboratory experiences, probable results were achieved. Probiotics were proven to arrive alive to its location surviving the digestive process. Moreover, it was shown that they are not affected by one of the factors that the author considered that could inhibit their action, alcohol consumption. Still, antibiotics were confirmed to affect bacteria depending on the strain and the type of antimicrobial drug used.

However, it must be emphasized the fact that the conclusions cannot be assured with certainty owing to the complex studies that should be carried out so as to reduce the possible errors.

Resum

En aquest treball s'analitza l'eficàcia dels probiòtics i, en conseqüència, la importància de la seva ingesta per a la microbiota.

Recentment, s'han elaborat nombrosos estudis demostrant els beneficis de la microbiota per a la nostra salut. El concepte de microbiota es definiria com el conjunt de microbis, sobretot bacteris, que trobem en el nostre organisme.

Aquest tema és difícil d'investigar degut a les dificultats per obtenir mostres representatives. Per aquest motiu, s'ha decidit enfocar la recerca cap a l'eficàcia dels probiòtics, ja que prenen un rol important en la preservació de la microbiota. Això, al seu torn, propicia innumerables beneficis per a la salut. Aquests beneficis seran descrits i estudiats de manera teòrica, així com la relació dels bacteris que trobem en els probiòtics amb la microbiota.

L'eficàcia de la ingesta dels probiòtics va ser contrastada realitzant nombroses experiències al laboratori tals com la verificació del fet de si aquests bacteris estan vius, si són capaços de sobreviure al procés digestiu i els possibles factors que els puguin afectar un cop hagin arribat al seu lloc d'acció.

Dels resultats obtinguts es dedueix que els bacteris que es troben en els probiòtics estan vius i que són capaços de sobreviure al procés digestiu. A més a més, es va observar el seu estat inalterable davant d'un factor que es va pensar que seria de risc, el consum d'alcohol. En canvi, es va provar que els antibiòtics sí que poden tenir efectes negatius sobre els probiòtics depenent del tipus d'antibiòtic i de la taxonomia del bacteri.

Cal destacar que els efectes sobre la salut que produeix la introducció d'aquests bacteris no van ser estudiats de manera pràctica, però sí teòrica. De manera que amb la realització d'aquestes experiències només es podria concloure que els bacteris que trobem vius en els probiòtics sobreviuen al procès digestiu i que no són inhibits pel consum d'alcohol, però sí per la ingesta de determinats antibiòtics.

Introduction

I have always been interested in biology and especially the field related to human health. This is what I told my tutor when she asked me the topic of my research. She sent me an article about the importance of the microbiota in humans which I found truly fascinating. This was the first time I had heard of that term but soon after, I was doing research on it.

It did not take much time for me to realize that an experimental part would be really difficult to be done owing to the limited possibilities I had to obtain the needed material.

There is an important relation between the intake of probiotics and the microbiota and that is the reason why I got into them. Scarcely did I know about probiotics before starting my work except from the fact that I had to take them once when I was given antibiotics.

First of all, I read some books and articles about the theme to get a general idea before starting my work. What really introduced me to the topic was reading a book called *"La digestión es la cuestión"* by Giulia Enders. Then, so as to have more and different references, many books in English taken from the UB were used as well as distinguished studies about the microbiota, probiotics and bacteria.

Afterwards, the objectives of the research were clearly stated:

1. To understand the importance of the microbiota for the human health.
2. To study the efficacy of probiotics.

While developing the main points, the possible laboratory experiences were thought. The first one is to check whether bacteria in probiotics are alive so that when they are ingested they are capable of carrying out a beneficial action. In order to arrive alive to the GIT, they must survive the digestive process which was actually the second experience. According to the general definition of a probiotics this should be accomplished due the fact that they must be alive so as to confer a health benefit.

However, what if there are factors that affect their living cycle once they have already arrived to their destination? The afore-said factors could be the intake of antibiotics and the consumption of alcohol. Concluding, the main objective of the laboratory experiences is to determine the efficacy of probiotics whilst in the theoretical part, it is exposed the reason why probiotics and the microbiota are important for health.

The work is organized so as to explain the general ideas and the basic concepts first and then the main points are exposed. First of all, a general idea of the microbiota is given, remarking the main points that will be needed for the understanding of the following sections. Then, the reader is introduced to the bacterial world, pointing out the main concepts so as to get a general idea. Afterwards, there are explained its main functions, its importance and some relevant studies which corroborate the concepts previously explained as well as the relation between its alterations and important illnesses. Then, probiotics are introduced to the reader not forgetting their definition and other important aspects. Finally, there are explained the laboratory experiences realized.

The main complications were deciding the objectives, finding some specific information and the fact of doing it in English. However, I thought that doing it in English would help me to consolidate the level

In spite of this, I learned a lot and it was a very enriching experience in many ways.

Content

1. Bacteria

Bacteria are **prokaryotic microorganisms** which do not have neither nucleous nor membranous organelles. Most of them are unicellular and their size oscillates between 1 to 10 µm compared to the eukaryotes size which is between 10 and 100 µm. They differ from the eukaryotic cells in organization, less metabolic variety, size and the fact that they do not have organelles.

They can colonize any environment thanks to their metabolic variety that will be explained in the following section.

Most bacteria have a rigid cell wall made up of peptidoglycan. They can have the shape of a sphere but they can also be lengthened, helical or curved. Many bacteria are mobile and they move by virtue of flagellums or other systems.

1.1. Bacterial metabolism

On the one hand, referring to the sources of carbon and energy there can be distinguished four different groups which will be exposed subsequently.

SOURCE OF ENERGY			
		Oxidation of molecules	Light
SOURCE OF CARBON	CO_2	**Chemoautotroph**	**Phototroph**
	Organic molecules	**Chemoheterotroph**	**Photoheterotroph**

Table 1. BACTERIAL METABOLISM-II

Esteller Pérez and others. (2010). *Biologia-2.* Barcelona: Vicens Vives

On the other hand, referring to their relation with the oxygen, five groups are distinguished. They will be exposed and briefly explained in the following table.

STRICT AEROBE	The **aerobic respiration** is carried out and as a consequence, oxygen is needed.
FACULTATIVE AEROBE	Both **aerobic and anaerobic respirations** are realized as well as fermentation in this type of bacteria. Oxygen is not strictly needed and as a result, they can live in a medium without it.
MICROAEROPHILIC	Microaerophilic bacteria carry out the **aerobic respiration** but cannot tolerate high levels of oxygen. Their enzymes are unstable unless there is **scant oxygen**.
STRICT ANAEROBE	Oxygen is a toxic gas for strict anaerobes and they effectuate the **anaerobic respiration** along with **fermentation** processes.
AEROTOLERANT ANAEROBE	Oxygen is tolerated but not used owing to the fact that **fermentation** is realized.

Table 2. BACTERIAL METABOLISM-I

Esteller Pérez and others. (2010). *Biologia-2.* Barcelona: Vicens Vives

1.2. Bacterial morphology

There are three main types of bacteria, although some may be star-shaped or square.

- **Coccus**: They have a spheric shape and they can appear isolated, in groups of two named diplococci or in cubic masses called sarcinas.
- **Bacillus**: They are lengthened bacteria and they have a cylindrical shape. They can be isolated, in groups of two called diplobacils or in a chain (estreptobacils).
- **Spiral**: They can be slightly curved and have three different shapes:
    - Comma shape called *vibrions*.
    - Helical rigid shape named *spirilla*.
    - Helical flexible shape known as *spirochaetes*.

Bacteria can appear individually or forming pairs, chains, clusters or other types of arrangements depending on their genus.

Some bacteria, in natural environments, can show themselves adhering to a surface forming **biofilms**. In that way, they appear in communities having a higher resistance to environmental factors. Biofilms are biological systems in which cells are connected to each other by means of a chemical communication system. Antimicrobials are believed to be a preventing tool against the formation of biofilms when applied to the surface on which they might be formed. Biofilms normally offer bacteria a protective barrier giving them resistance to antibiotics.

1.3. Structure

1.3.1. General structure

Bacteria are organisms with a really **simple cellular structure**. Their genetic material is not delimited by a nuclear membrane and as a consequence, bacteria do not have a nucleus. In the cytoplasm, ribosomes and reserve substances can be found but there are not any organelles delimited by membranes.

The cytoplasm is surrounded by the plasma membrane, formed of lipids and proteins and the cellular wall which is made up of peptidoglycan. The peptidoglycan is a carbohydrate and a protein complex. Some bacteria also have a second membrane that surrounds the cell wall. They can also have a polysaccharide layer and some other external elements such as flagella, fimbrae and pili which have the role of propelling bacteria.

Some bacteria secrete mucosae layers and depending on their rigidity and relation with the cell, they can be named capsule or glycocalyx. Both can be made up of polysaccharides and, in some cases, of proteins too. Most of them are made inside the cell and then secreted to the external surface.

In case of not being an organized structured and slightly unattached to the cell wall, it is called slime layer.

1.3.2. Internal structure

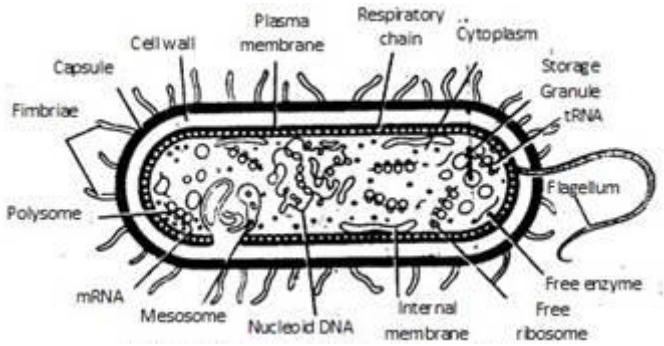


Figure 1. STRUCTURE OF A BACTERIAL CELL

http:// swww.studyadda.com/notes/11th-class/biology/kingdom-monera/structure-of-bacteria/9545

1.3.2.1. Cytoplasm

It refers to the **substance** contained inside the plasma membrane which is approximately 80% water. It also embraces **proteins** (enzymes), carbohydrates, lipids, inorganic ions and low-molecular-weight compounds. The main components of it that will be analyzed in the subsequent sections are: the cytosol, ribosomes, inclusions and the nucleoid. Prokaryotic cells have a cytoskeleton which does not differ much from the one in eukaryotic cells and it is a collection of fibers that provide the cell a defined and stabilized structure.

1.3.2.2. Nucleoid

The nucleoid is where the **bacterial DNA** is contained and it is called the bacterial chromosome. It contains all the genetic information required for the bacteria's functions and structure. The referred bacterial chromosome is a single double-stranded DNA fragment which is normally circular, long and rolled up. They differ from the eukaryotic chromosomes because they do not have histones and they are not surrounded by a membrane. The percentage of volume that it occupies varies with the functions of each cell and it can represent up to the 20% of the total volume.

Nevertheless, small and normally circular DNA molecules are usually contained in the exterior of the aforementioned bacterial chromosome. They are called plasmids and they may contain crucial genes for antibiotic resistance. When being transferred to other cells, the antibiotic resistance is extended between bacteria.

1.3.2.3. Inclusions

Inclusions are known to be **reserve deposits** that can be found in the cytoplasm in which nutrients may be accumulated. They can sometimes be used for the identification of different bacterial strains due to the fact that some of them are typical of certain genus.

1.3.2.4. Ribosomes

The ribosomes are the only organelles in the bacterial cytosol and their function is the **protein synthesis**. They can also be found in eukaryotic cells although they differ in size because the ones in prokaryotic cells are smaller.

In the **Antimicrobial drugs** section it will explained how antibiotics can kill bacteria by affecting ribosomes and therefore the protein synthesis.

1.3.2.5. Plasma membrane

It is an osmotic **barrier** which surrounds the cytosol and it is located in the interior of the cell. Its main compounds are **phospholipids** and some proteins. The referred phospholipids are disposed in parallel rows and that is why it is called the lipid bilayer. There is a presence of membrane proteins too and this combination of proteins and phospholipids is called the fluid mosaic model. It has some internal folds called mesosomas and it is similar to the cell membrane in eukaryotic cells.

Mesosomas are believed not to be cell structures and they are described as respiratory enzymes. They have been compared in some studies with mithocondrial cristae found in eukaryotic cells.

The cell membrane hosts multiple complex metabolic processes due to the absence of organelles and it has many functions.

In the **Antimicrobial drugs** section it will be discussed how the antimicrobial agents do affect the plasma membrane.

1.3.2.6. Bacterial wall

Surrounding the plasma membrane there is the bacterial wall which shapes the cell. Its composition and structure vary with the genus.

The main components of it are the chains of oligosaccharides which form a network of a macromolecule named **peptidoglycan** (which can be known as *murein*).

There are two types of bacterial cell wall which divide bacteria into two general groups: **Gram-positive** bacteria and **Gram-negative** bacteria.

Overall, nearly all bacteria are included in these two groups with the exception of mollicutes and archaebacteria. Mollicutes, as opposed to other bacteria, do completely lack walls. On the contrary, archaebacteria may lack walls or they may have walls which do not contain the usual murein and they slightly diverge from the typical prokaryotic cell. Their wall is not alike the one in Gram-positive or Gram-negative bacteria owing to its composition made up of a compound which differ slightly from the peptidoglycan, the pseudomurein, and the fact that it is surrounded by an external sheath. They do not have internal membranes but a dense ribosomal packing of the cytosol.

- *Gram positive bacteria*

They have a rigid and **thick bacterial wall** made up of peptidoglycan layers and it can appear to have even 40 of them. However, they do not have either an external membrane or periplasmic space. The different peptidoglycan chains join together by mean of the peptide bond forming the exoskeleton which gives bacteria consistency and a special shape for the replication.

Besides, the nutrients such as sugars or amino acids can go through it easily due to its hydrophilic structure.

- *Gram negative bacteria*

They have only one or a few **thinner bacterial walls** made up of murein (peptidoglycan) which is surrounded by an external **lipidic membrane** that contains lipoproteins, liposaccharides and porins. Therefore, both types of bacteria have the same bacterial wall composition but they differ in the thickness of it.

This outer membrane has the capacity to resist detrimental chemicals and it differs from the typical biological membranes.

This external lipidic membrane has channels that are composed of proteins known as porins. Porin channels allow the passive diffusion of hydrophilic compounds such as sugars, amino acids and specific ions.

The space between the external plasma membrane and the lipid bilayer is called periplasmic space.

A comparison between both types of bacterial walls can be seen in the following figure.

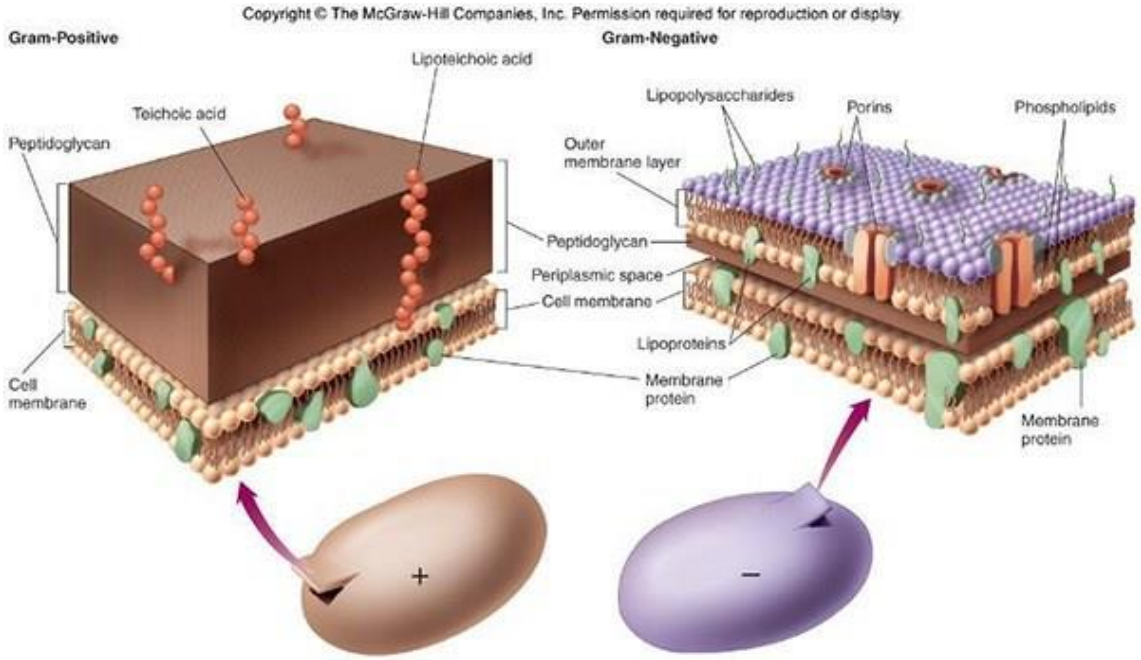


Figure 2. GRAM-POSITIVE / GRAM-NEGATIVE BACTERIAL WALL

The McGraw-Hill Companies, Inc.

1.4. Bacterial reproduction, genetic transfer and recombination

Bacteria reproduce by **asexual methods** and as a consequence they inherit identical genes. The growing and the reproduction by cellular division consist of growing until a specific size and then the division by scissiparity or fission is started. This process starts with the duplication of the DNA strand. This process is called **binary fission**.

1. The origin of replication is marked in green and there is where the process begins.
2. The DNA starts its duplication.
3. The mesosomas start the separation of the two DNA strands.
4. The cytoplasmic membrane elongates and therefore, it separates the two DNA copies. The cytokinesis starts and the cytoplasm is divided into the two different cells meanwhile the cell wall and the plasma membrane start constricting. Lastly, the bacterial cell wall grows until it forms a septum and the two cells separate.

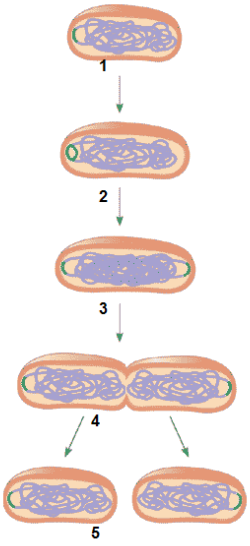


Figure 3. BINARY FISSION

http://willa.me/binary-fission-facts-check/

They can evolve through **mutations**, which introduce DNA changes, and through horizontal gene transfer and the following genetic recombination. This horizontal gene transfer is not related to reproduction and it can be produced between bacteria from different species. It is based on the introduction to the bacterial chromosome a fragment of a foreigner DNA called donor DNA, this process is known as crossing over. When it has been done, the bacterium carries a fragment of the donor's DNA. It can be done through three different mechanics: transformation, transduction and conjugation.

- **Transformation**: It is a process that makes bacteria able to introduce an exogenous gene* called transgene, which comes from the lysis of other bacteria. It will be introduced and replace a piece of the bacterial genome. This procedure is named recombination.
- **Transduction**: It consists in the gene transfer between bacteria done through a bacteriophage which is a virus that affects mainly bacteria and it carries pieces of the DNA of the last parasitized bacteria.
- **Conjugation**: It is a method in which the DNA is transferred by direct cell-to-cell contact by means of a pilus between two cells.

1.5. Bacterial growth

In some cases, usually when cultivating bacteria in a liquid growth medium, it is possible to determine the quite exact number of colonies which have been and will be growing over the time. By virtue of this, it can be plot a bacterial growth curve that will reflect the bacterial growth over time. It has four different phases of growth which are called the lag phase, the log phase, the stationary phase and the death phase.

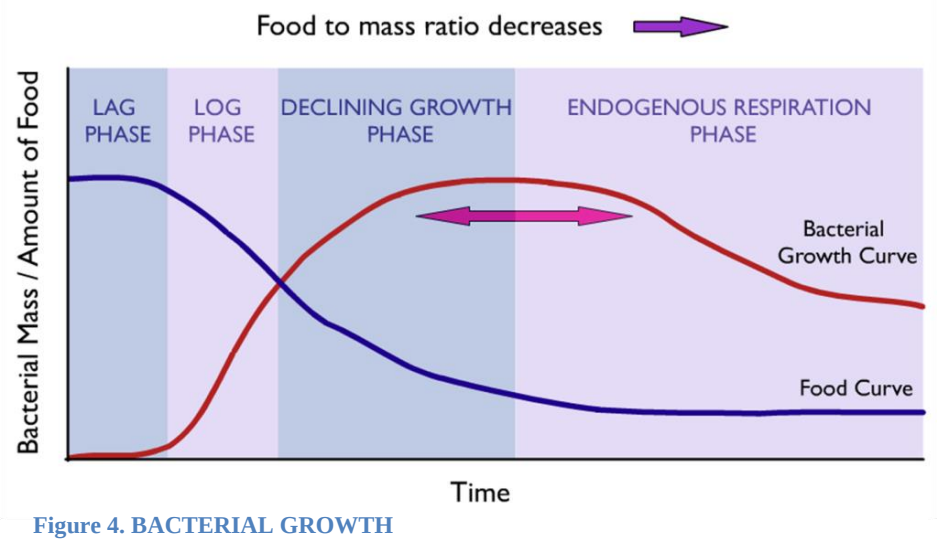


Figure 4. BACTERIAL GROWTH

http://www.ebsbiowizard.com/biological-growth-curve-in-aerated-stabilization-basins-765/

In the previous image, it can be seen the bacterial growth and the decreasing amount of food over time. It can be appreciated that bacteria in the log phase increase by a factor of two due to the fact that when a cell reproduces by binary fission the results are two daughter cells. However, when arriving to the stationary phase, which is marked with an arrow in the graphic, it coincides with the only period of time when the function is flat-lined. Nevertheless, the last part of the graphic which can be called death phase, the gradient of the line is declining.

In the following sections, each phase will be analyzed in detail as well as their duration and processes which undergo the cells in that time.

1.5.1. The lag phase

This period remains for 1 hour or various days. In it, there is **no division** or in any case, just in little proportions. This can be explained because bacteria do not reproduce immediately in a new medium. In spite of the fact that there is no cell division, there is a high metabolic activity including the synthesis of enzymes and molecules.

1.5.2. The log phase

It may be called exponential growth phase and it starts with the **division** of bacteria. The bacterial cells divide in a steady rate which depends on the incubation conditions and the conditions of the medium used. The logarithmic plot during this phase is linear owing to the constant generation time. The referred generation time is defined as the time needed to complete a generation or as the rate of **exponential growth**.

The log phase is the period of time in which bacteria are ongoing the highest levels of metabolic activity.

1.5.3. The stationary phase

This phase can be known as the **equilibrium phase** in which the growth rate decreases and the number of death is likened to the quantity of new cells instead of the cells dividing and resulting in an excessive population.

There is not a clear explanation for this but there are some factors which may the cause. For example: the exhaustion of nutrients, the changes of the pH levels or the accumulation of profitless end-product.

1.5.4. The death phase

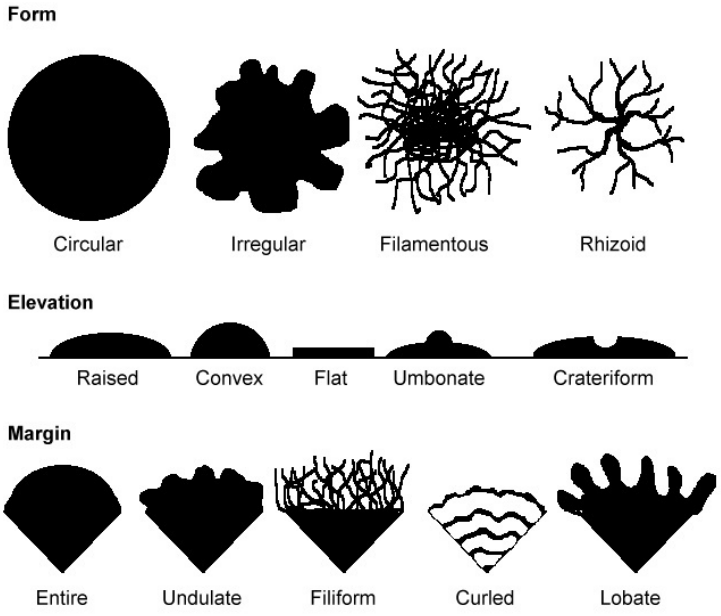
It can be named logarithmic decline phase and it begins the moment when the number of **deaths** passes the amount of new cells originated. This phase goes on until the number of colonies represents just a little part of the amount in the previous phases or until the population ceases to exist.

1.6. Bacterial colonies

A bacterial colony is defined as a **conglomeration of bacteria** usually being the clone of a previous one being all genetically identical. They appear on or within the surface of a medium. It offers bacteria many advantages such as the possibility of mutations which could make them resistant to antibiotics.

Each bacterial colony has different characteristics that will be exposed next and it depends on the strain forming it.

1.6.1. Characteristics



Different bacterial characteristics when forming a colony can be used in order to identify distinct bacterial strains. The referred characteristics can be seen in this figure but also the color and the size of it must be considered when analyzing them.

Figure 5. BACTERIAL COLONIES CHARACTERISTICS

https://www.science buddies.org/

1.7. *Taxonomy of bacteria*

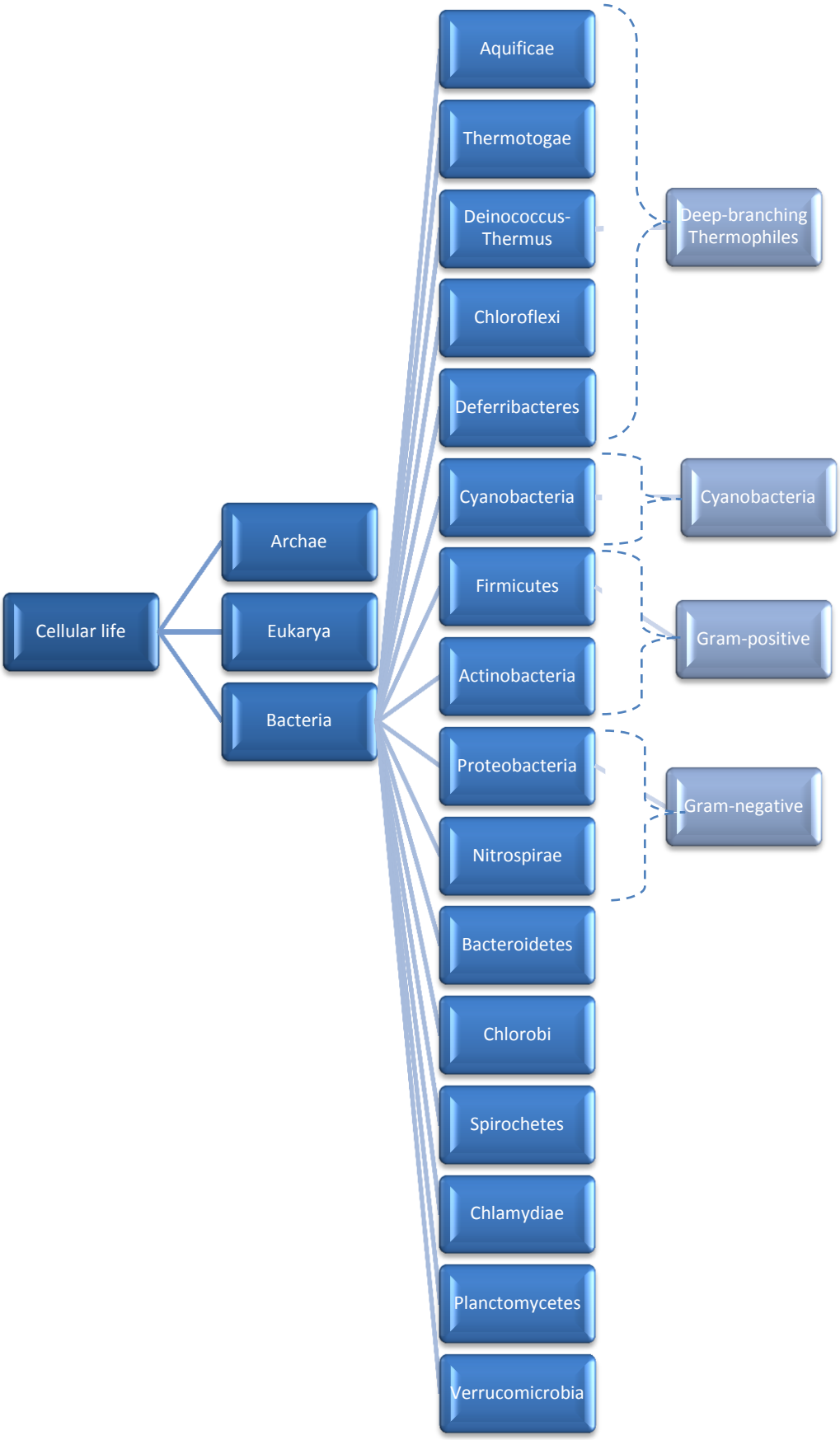


Table 3. BACTERIAL TAXONOMY

2. The microbiota

The microbiota is the collection of microbial species, mainly **bacteria**, which are found in regions where they can have access through one or more body orifices. In the GIT they find their optimal temperature for life and they act like an organ. The normal microbiota has many health benefits such as the production of needed vitamins (vitamin K and some B vitamins) and the prevention of the growth of detrimental microbes.

Moreover, the relation between us and our microbiota is based on the principles of mutualism owing to the fact that we offer them the temperature that they require and they provide us with many health benefits that will be explained in the **Functions of the microbiota** section.

When talking about our microbiota and us, we must refer to a **symbiotic relation** due to the mutual benefits obtained. However, the aforementioned balanced relation can break down causing an endogenous or opportunistic infection.

These abovementioned communities of bacteria have their own genes, called microbioma which is sharply over the human genome. While we have got from 20.000 to 25.000 genes, the microbial communities colonizing the human GIT have approximately 3.3 million genes.

As it has been exposed, the microbiota is the collection of bacteria which is found in our different body sides. For this reason, the following sections will introduce the reader to the bacterial world so as to have the basic knowledge to understand the work itself.

2.1. Acquisition of the microbiota

This aforesaid collection of microbial species is acquired immediately **after birth** during the labor.

When culturing and analyzing a sample of the newborn's stomach, the species found resemble the ones found in the mother's cervical and vaginal microbiota. Moreover, species from the mother's GIT can also be present such as Bacteroides and E. coli.

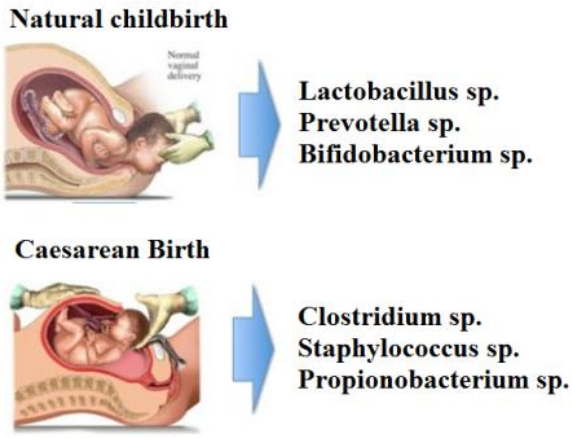


Figure 6. ACQUISITION OF THE MICROBIOTA DURING LABOR

Arenas, M. andothers. (2015). *All youneed is biology.* https://allyouneedisbiology.wordpress.com/

Other potential colonizers are microorganisms detected on the mother's skin and saliva as well as the ones present on the environment.

In case of a caesarean birth, a gauze impregnated with the mother's vaginal microbiota should be applied to the neonate. In that way, the possibilities of the newborn being colonized by environmental microorganisms or the ones coming from the hospital staff are reduced. The problem with the aforesaid microbes of the hospital staff is that they are resistant to one or more antibiotics, causing problems to the neonate in case of being treated with antibiotics later in his life.

During their first days, they may get exposed to different environments and individuals apart from their mother and therefore they come into contact with their microbes.

However, during their first weeks of life, it appears a key factor which is going to make a difference in their microbiota. This key factor is whether they are BF or FF.

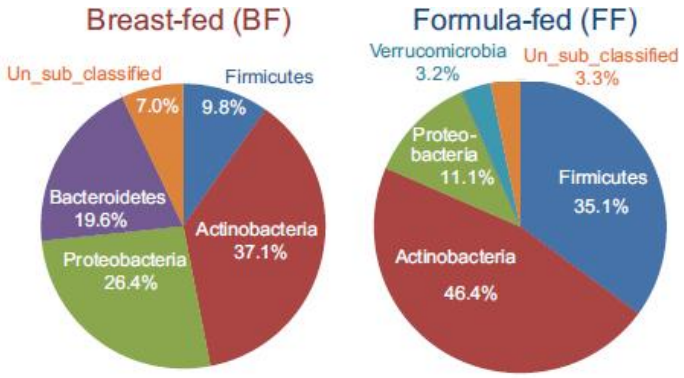


Figure 7. DIFFERENCE BETWEEN THE MICROBIOTA OF A BF OR FF

Donovan et al. Advances in Nutrition. 2012

The difference in their microbiota can be appreciated when analyzing these two graphics.

2.2. Changes throughout life

When being an infant, there are two different factors which change the microbiota. The aforesaid factors are **antibiotic treatment** and **malnutrition**. As a consequence, there can be distinguished three different types of microbiota.

When being treated with antibiotics, the amount of proteobacteria is lowered while there is an absence of actinobacteria.

On the other hand, in case of malnutrition there is a high increase of proteobacteria.

When growing old, the percentage of firmicutes increases whilst the amount of bacteroidetes tends to decrease sharply. In case of obesity, the number of proteobacteria grows.

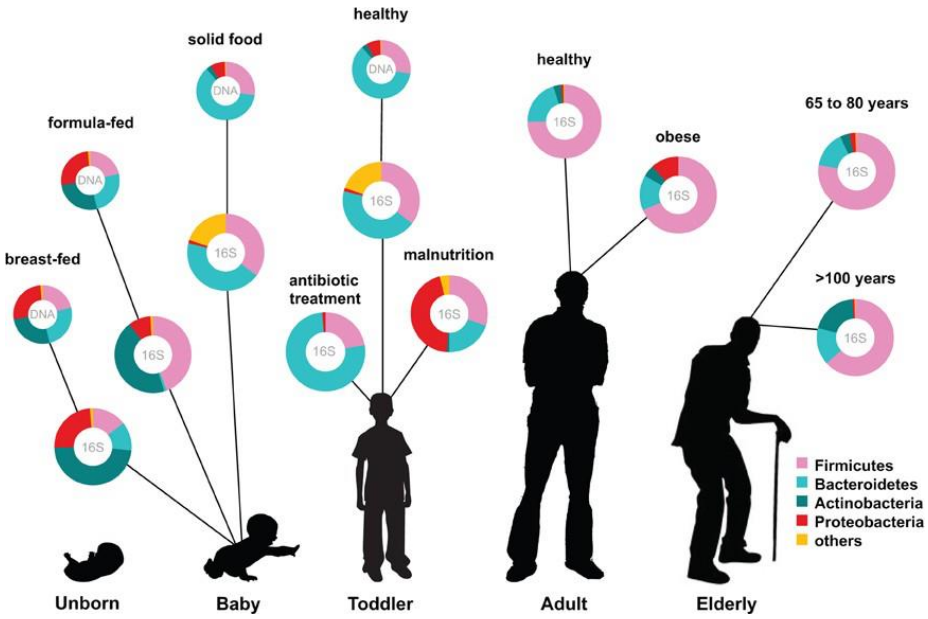


Figure 8. CHANGES OF THE MICRBIOTA

Ottman et al. Front Cell Infect Microbiol. 2012

In elderly people, the amount of actinobacteria augments while the number of firmicutes and bacteroidetes is lowered.

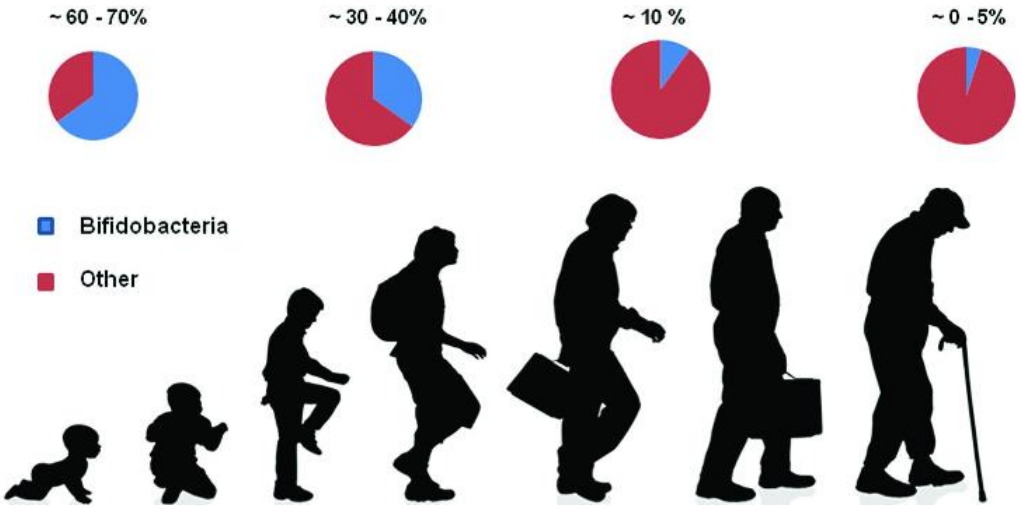


Figure 9. LEVELS OF BIF. DURING LIFE

When talking about the presence of Bif. bacteria it must be analysed its decrease as we age. Going from a 60-70% to a 0-5%. As it has been said previously, Bif. confer many health benefits and therefore, its decrease could increment the possibilities of desease caused by microbes.

2.3. *The microbiota in different body sites*

Microbiota can be found in the urogenital, respiratory and GI tracts, in the mouth, pharynx, vagina, skin… Bacteria strains vary depending on the body site. The only body sites which are sterile in humans are the circulatory system, the pleura, meninges, the pericardium, the peritoneum and the uterus.

The different strains found in the diverse body sites are presented in figure 10.

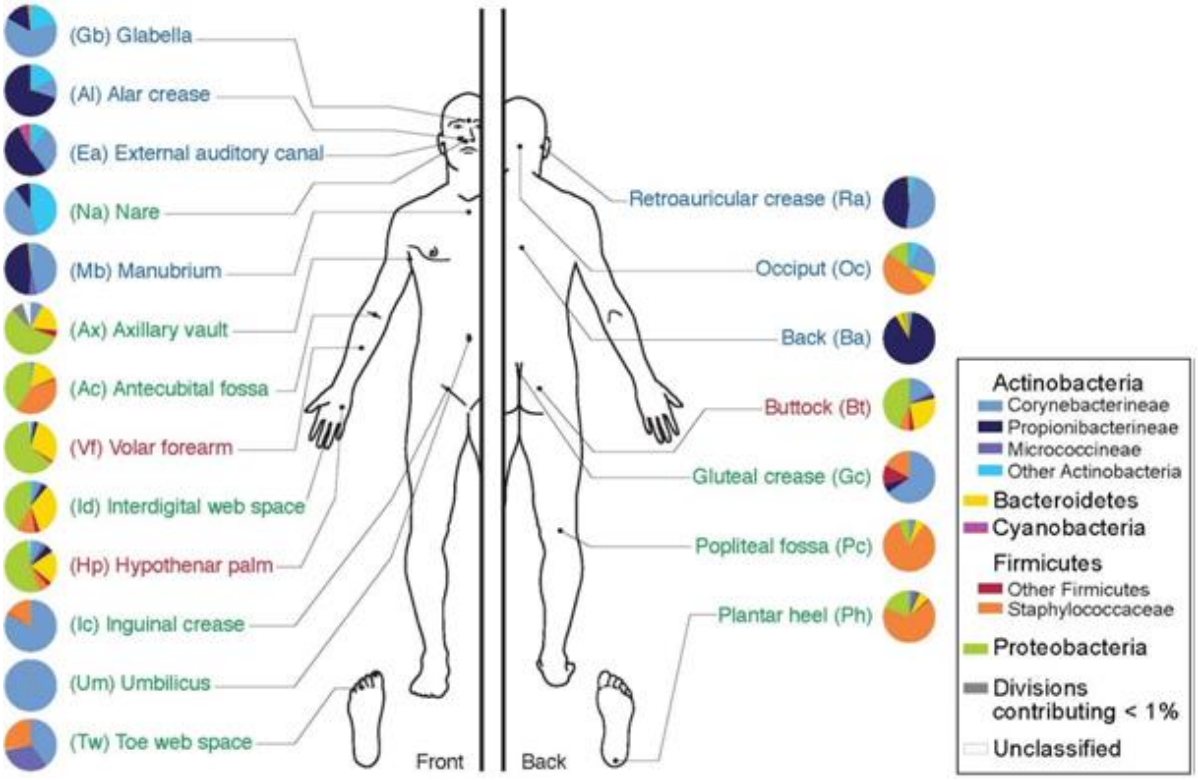


Figure 10. MICROBIOTA IN DIFFERENT BODY SITES

Griceand Segre. Nat. Rev. Microbiol. 2011

The stomach and the first two-thirds of the small intestinal tract are the body sites that have less quantity of microbes even though nutrient availability is higher than in the other sites. The human stomach is lined with a glandular mucosa which lactobacilli can hardly colonize.

The microbiota found in an specific body site does not highly differ from the one found in other human beings in spite of the differences in the climate, the diet, the hygiene measure and the lifestyle habits.

2.4. Functions of the microbiota

The microbiota has many health benefits for us that will be explained subsequently.

- **Metabolic and digestive functions**: Bacteria belonging to the microbiota are in charge of the fermentation of the indigested food. Thanks to that, energy is obtained. The microbiota also takes advantage of the undigested nutrients. This fermentation of indigested compounds can provoke discomfort due to the gases generated.
- **Trophic function**: The aforementioned bacteria take a part in stimulating the immune system and the neuroendocrin cells located along the GIT. This is thanks to the antigens that they provide us with and the secretion of specific factors, which activate the immune system.
- **Production of vitamins** that we need such as the vitamin K and some types of vitamin B such as B3 and B6. The supply of amino acids is also considered to be an important function. LAB found in the GIT when producing lactic acid are contributing to the absorption of minerals such as calcium, iron and phosphor.
- **Defense function**: It has a bacteriostatic capacity which acts as a defense barrier. Thanks to the bacteria found in the GIT it is generated a competition between pathogenic and autochthonous bacteria for food and space which impedes their growing.

The microbiota has been related to different illnesses which will be described subsequently:

On the one hand, recent studies have shown a relation between **obesity** and the microbiota. It is affirmed that thin people tend to have higher levels of bacteroidetes while obese people are believed to have more firmicutes even if both groups have the same diet. This was proven with microbiota transplants on mice which confirmed this theory.

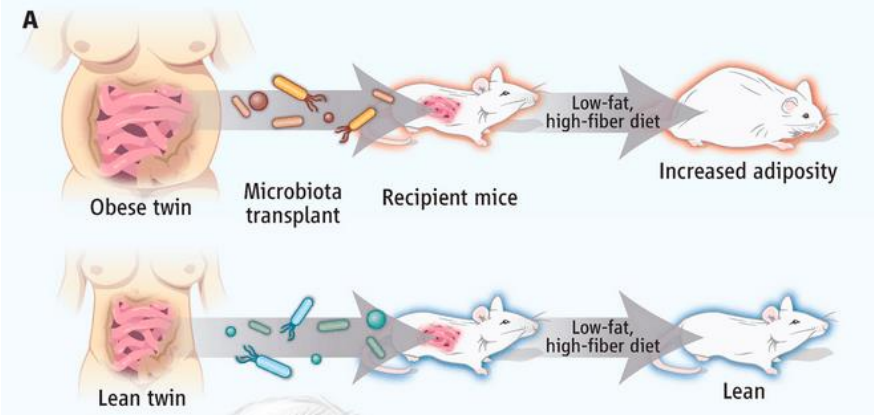


Figure 11. MICROBIOTA TRANSPLANTS ON MICE

Ridarura et al. Science. 2013

Moreover, it has also been associated with **diabetes type II** due to the fact that the composition of it is alterated.

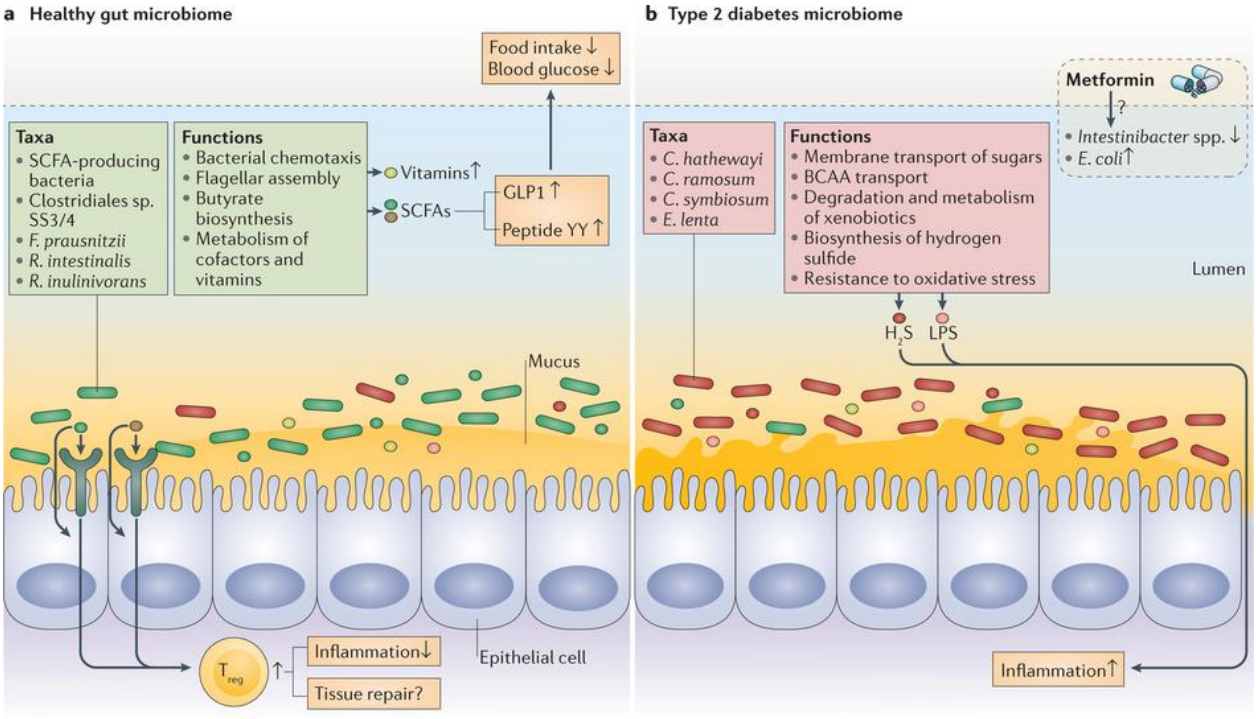


Figure 12. MICROBIOTA AND BIABETES TYPE II

Wang and Jia. Nat. Rev. Microb. 2016

Furthermore, a relation between it and **cancer** has also been discovered as well as between **psychic diseases**. This is thanks to the connection between the brain and the intestine by virtue of the vagus nerve and spinal pathways. Besides there can also be found neurotransmitters in the GIT. This is illustrated in the subsequent image.

The possibilities of preventing illnesses as the ones exposed previously just by taking care of our microbiota are being studied but they seem to be reachable.

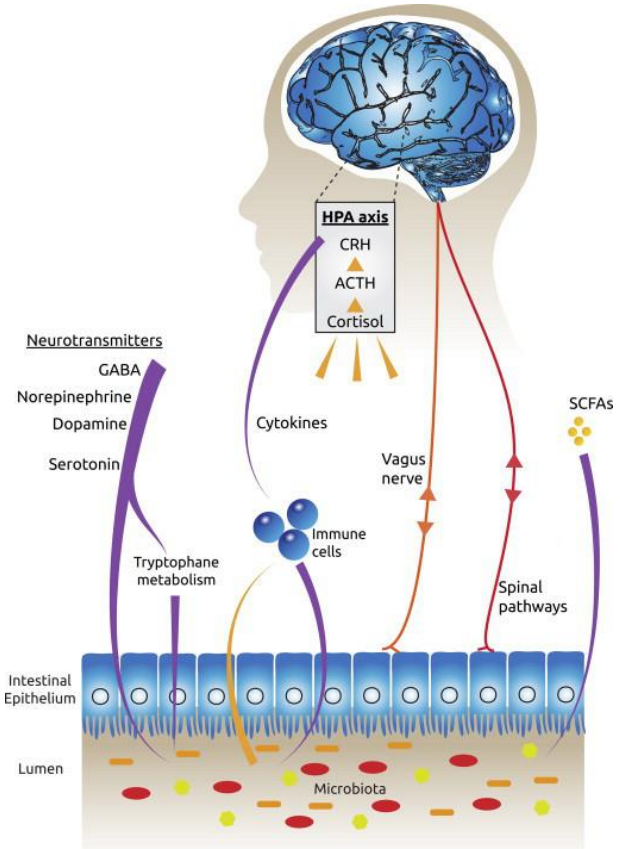


Figure 13. MICROBIOTA AND PSYCHIC DISEASES

Dinan et al. J. Psychiatr. Res. 2015

2.5. Alterations of the microbiota

Bacteria found in the GIT are susceptible to different factors which can affect the normal microbiota. The referred factors will be subsequently exposed and some may be analyzed in detail in the following section.

- The **food** ingested and our diet, have an important role in determining the microbiota.

- **Alcohol** consumption can have a decisive performance in the modification of the microbiota. It will be explained in depth in the following section. Alcohol has been proven not to arrive to the colon which is the body site where most bacteria are located. Subsequently, it will be exposed meticulously how is alcohol absorbed when ingested.

Soon after the ingestion of alcohol has taken place, the 20% of it is rapidly absorbed into the bloodstream. From the blood it is distributed to the different tissues and fluids in the body depending on the relative water content, the rate of blood and the tissue mass.

In the liver there can be found in a largest extent two of the enzymes that are in charge of the transformation of alcohol to acetaldehyde. The referred enzymes are called ADH and CYP2E1. After that, acetaldehyde is turned into acetic acid which is metabolized to carbon dioxide, water or fatty acids.

However, a small quantity of alcohol is not metabolized and therefore it is excreted through sweat, saliva, urine and breath.

In the practical part, some experiments will be carried out which will demonstrate whether bacteria can survive high alcohol levels or not. Little research has been done about the possibilities of the microbiota being altered by alcohol consumption. However, some studies have shown that bacteria may tend to overgrow when being in presence of it, especially Gram-negative bacteria. Furthermore, the overgrowth of the referred type of bacteria is considered prejudicial due to the accumulation of endotoxins.

- **Age** is also important when talking about modifications in the microbiota as it has been explained in the **Changes throughout life** section.

- Particles found in the **air** can be crucial in the composition of the human microbiota.

- The modification of the microbiota may be caused by **stress** and **fatigue**.

- **Sedentariness** can be a possible reason of an alteration of the microbiota.

- **Smoking** is a detrimental habit for the GIT health but not only that but the fact of being exposed to any type of smoke continuously.

- **Antibiotics** and medicines can change the balance of bacteria found in the GIT. Antibiotics have long been used to treat infections but little did they imagine that they would also affect the beneficial bacteria found in the GIT. In the case of beneficial bacteria being killed by the antibiotics, other bacteria can start overgrowing. These bacteria are called **opportunistic bacteria** and they can affect us in a negative way. These referred opportunistic bacteria can cause for example diarrhea. Many illnesses that are continuously increasing nowadays such as asthma and allergies have been associated to the decreasing variety in the intestinal ecosystem.

Antibiotics also contribute to the **loss of bacterial diversity**.

- Recurrent **infections** can be the cause of an imbalance in the microbiota.

2.6. Bacteria in the microbiota

In the present section there will be exposed the bacterial strains that can be found in the human microbiota and some of their main characteristics.

• ACTINOBACTERIA
  ○ Corynebacterineae
  ○ Propionibacterineae
  ○ Micrococcineae
  ○ Other
	It is a group of **Gram-positive** bacteria and they develop the **aerobic** respiration. Some of them may be pathogenic strains.

• BACTEROIDETES	**Gram-negative** and rod-shaped bacteria which can be found in the **mouth** and the **GIT**. In some cases, they are opportunist pathogens.
• CYANOBACTERIA	They are also **Gram-negative** cells but they do the **oxygenic photosynthesis**.

• FIRMICUTES
  ○ Lactobacillus
  ○ Staphylococcus
  ○ Streptococcus
  ○ Other
	This bacterial strain has been proved to be **Gram-positive** in most cases. There have been evidences that show that some of them may be pathogenic.

• FUSOBACTERIA	They are believed to stain as **Gram-negative** bacteria and they can be found in the human **mouth** and **GIT**. These taxa are rod-shaped and some may be pathogenic.
• PROTEOBACTERIA	Their bacterial wall defines them as **Gram-negative bacteria** and they include many pathogens too.

Table 4. BACTERIA IN THE MICROBIOTA

In the following sections, some bacterial strains will be exposed in depth due to the fact that they were used for the experiences. Therefore, the information given will be profitable when carrying out the experiments.

2.6.1. Lactobacillus

It is a genus of bacteria which are known to be Gram-positive, chemo-organotrophic*, fermentative, microaerophilic and catalase-negative*.

They can occur as rods and coccobacilli*.

These taxa belong to the LAB. This name comes from the fact that bacteria pertaining to this group have the ability to produce lactic acid as a result of the fermentation of carbohydrates.

This genus is said to be important for human health due to its role in food and supplies conservation and generation. This aforesaid food production refers to the one that requires lactic acid fermentation. One instance could be some dairy products such as yoghurt, cheese, fermented vegetables, fermented meats, wine and sourdough bread. Food which has been fermented by a lactic fermentation process is considered to be beneficial for the consumer.

Moreover, some strains do have probiotic properties. LAB are also known as fermentative bacteria that impede the growth of non-acid-tolerant bacteria which were considered to be detrimental by Elie Metchnikoff.

They are practically ubiquitous that is to say that they can be detected in environments with carbohydrates, such as food. Furthermore, they can also be found in respiratory, GI and genital tracts of animals and humans, and in residual waters as well as in plant material.

- L. acidophilus La-14

It is one of the dominant autochthonous *L.* species in humans. They are widely distributed and found in milk and dairy products and as commensals in the alimentary tract of mammals. They are used to make 'acido-philus milk' and are associated with dental caries in humans. They can be detected in faeces, in the oral cavity, vagina and the GIT. Colonies of this organism on agar media are described as either 'feathery' or 'crab-like'.

- L. plantarum Lp-115

It is widely distributed on plants, alive and dead, and is one of the organisms responsible for fermentation in pickle. It can be found in faeces, in the oral cavity and in food. It gives poor growth on agar.

It is also known to be the microorganism that produces sauerkraut from the starting material cabbage. It can be used in the processes of making sourdough bread, meat products and wine.

- L. paracasei Lpc-37

It is a heterofermentative rod which is found in the GIT and in fermented vegetables, milk and food.

Its benefits are multiple such as its ability to reduce antibiotic effects on human microbiota and therefore it stabilizes the microbiota during and after antibiotic treatment.

It has been confirmed its resistance to many antibiotics.

- L. gasseri

It has been said to be beneficial for the microbiota due to the fact that it increases the levels of Bif. which at their turn offer a health benefit. It has been proven to positively contribute to the good realization of the microbiota functions as well as the prevention of its alterations.

2.6.2. Bifidobacteria

Bif. bacteria have also been claimed to be important for human nutrition the same way as L.

Regarding to its shape, they are small polymorphic branched rods. They can appear individually, in chains or in clumps.

They predominate in the faeces of breast-fed infants, but they are also commensal in the adult bowels, mouth and vagina.

They are generally obligate anaerobes even though they can also grow in carbon-dioxide-enriched air environments. They are catalase-negative with some exceptions. They produce acid from glucose. Besides, they are capable of fermenting sugars as well as hydrolyzing a wide range of polysaccharides. Bif. have been proven to be acid-tolerant yet no acidophilic.

They have the ability to live in environments with temperatures from 20º to 49.5º.
In addition, their pH range for growth is from 4.0 to 8.5.
Furthermore, they are believed to be a source of vitamins.
In relation to the benefits they offer, the antimicrobial compounds they produce must be referred as well as the low pH they generate when producing acid from glucose and in that way inhibiting partially microbial growth.
Bif. may be used as probiotics due to their beneficial effects on human health that will be exposed consequently.
First of all, they impede the attachment of pathogenic microbes to the IECs. Moreover, as their end-products tend to be acidic, they inhibit microbial growth. In addition, antimicrobial compounds are also produced by Bif. bacteria which result into the death of some pathogenic strains as well as the inhibition of some specific microbes.

- Bif. lactis BI-04

Bif. lactis is believed to confer a health benefit when ingested due to the fact that it has been proven its resistance to the conditions in the GIT such as the acid and bile. Moreover, it has an excellent adhesion to the IECs. In addition it can be used to balance the microbiota after the intake of antibiotics. Besides these advantages, it also has a role in stimulating the immune system in specific ways.

- Bif. bifidum

Bif. bifidum is present in the oral cavity not forgetting its presence in the GIT. It can also be found to a lesser extent in the vagina.

- Bif. longum

Bif. longum is also detected in the GIT and the oral cavity the same way as the Bif. bifidum. Recent studies have shown their adaptation to the colon environment due to the fact that it has enzymes which can break down plant polymers which have not been digested in the small intestine.

3. The GIT

The digestive system consists of the GIT and the teeth, tongue, salivary glands, liver, gallbladder and pancreas which are known as the accessory digestive organs. Their function is to break down the ingested food into small molecules that will be absorbed later.

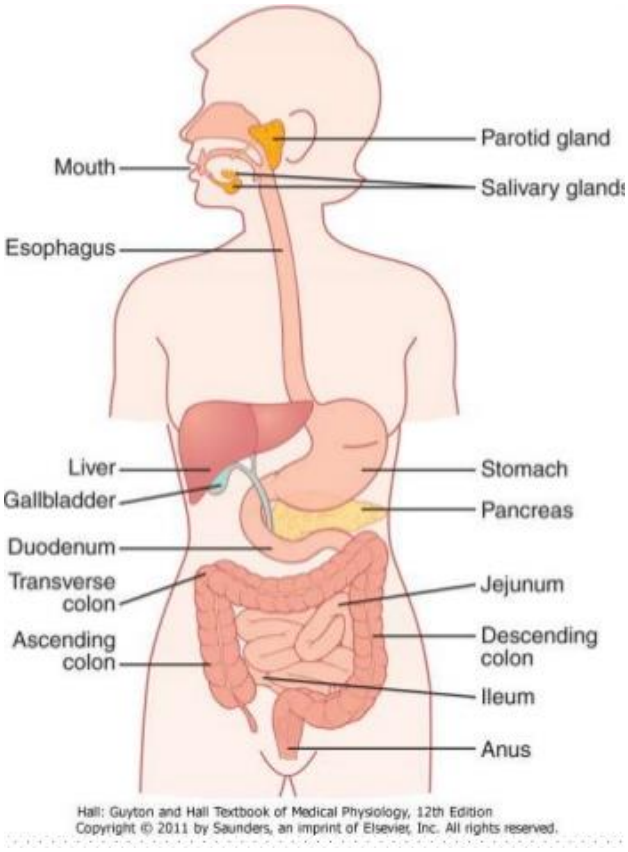


Figure 14. THE GIT

Guyton and Hall Textbook of medical physiology. 12th edition

The GIT is physically a large and continuous tube comprising four layers of tissue which includes the oral cavity, the oroharynx, the larynopharynx (which is also a part of the respiratory system), the oesophagus, the stomach and the small and large intestine. Due to the different functions they carry out, their anatomy and location, each one provides a specific environment for a particular microbiota with different bacterial strains. However, the microbiota found in the oral cavity does highly differ from the rest due to some special conditions that it offers. These features include high levels of oxygen, a complex anatomy and non-shedding surfaces such as teeth which enhance the formation of biofilms.

On the other hand, the other regions of the GIT have a lower diversity of bacterial strains and do not include non-shedding surfaces. The regions which are found below the stomach are mainly anaerobic whilst the regions within the large intestine are composed of a lumen which is normally filled with materials.

3.1. Autochthonous microbiota of the GIT

It cannot be assured with certainty the composition of the microbiota due to the difficulties in obtaining the samples.

However, the analysis that have been done show a huge **variety** of species that seems to be from 500 to 1.000 different species.

Moreover, a difficulty added is that the 20-60% of the microbiota cannot be cultivated owing to their anaerobic character. Therefore, genotypic identification methods are recurred.

4. The digestive process

First, the digestive process will be exposed and afterwards, the enzymes and compounds which have a role in it.

MOUTH	• **Chewing** of the ingested food and the following **insalivation** of the referred aliments. • Saliva contains an enzyme called amylase which is in charge of the partial hydrolysis of the starch to maltose. In this moment, the quimic digestion is started. • Closing of the epiglottis.
ESOPHAGUS	• Execution of the **peristaltic movements** which make the bolus progress.
STOMACH	• The bolus enters the stomach through the cardia. • It has a major surface thanks to the rugae. • The cells located in the stomach segregate the **gastric acid** whose components will be exposed in the following section. • Mixture of the components thanks to the muscular movements. • Way out through the pylorus.

SMALL INTESTINE	• It is where the major **absorption of the nutrients** is produced. • In the duodenum the bile is released from the liver and the **pancreatic juice** from the pancreas. • The nutrients that have been absorbed pass through the lumen to the **lymph** and the **blood**.
LARGE INTESTINE	• The chyle which has not been absorbed in the small intestine continues to the large intestine. • Throughout its route through the large bowel, the **absorption of water and mineral salts**. • **Bacteria** in the GIT are in charge of producing some reactions in which vitamins may be produced and some molecules might be metabolized. They take advantage of the molecules which have not been digested so as to obtain energy.
ANUS	• The rectum accumulates the **faeces** until the nervous stimulation initiates the **defecation** through the anal canal.

Table 5. DIGESTIVE PROCESS

Subsequently, the enzymes and the components which have a role in the digestive process will be exposed as well as their function.

GASTRIC JUICE	BILE	PANCREATIC JUICE
• **Pepsinogen**: When being in presence of the hydrochloric acid, it is transformed into pepsin and it turns proteids into proteoses and peptone. • **Hydrochloric acid**: It acts as an antiseptic killing bacterium except specific strains which can deal with low pH range. Its principal function is to reduce food to the maximum. • **Rennet**: Its purpose is having a role in digestion but no further details have been discovered yet.	• **Water:** It represents the 95% of the bile. There are dissolved the solid constituents in it. • **Bile salts:** Sodium salts which help in the emulsification of fats. • **Bile acids:** They are crucial for the digestion and absorption of fats. • **Cholesterol:** Bile acids are synthesized from it.	• **Bicarbonate:** It neutralizes the chymus. • **Amylase:** Its function is to be in charge of the hydrolysis of starch to maltose. • **Maltase:** It is responsible of the hydrolysis of maltose to glucose. • **Steapsin (pancreatic lipase):** Their function is to hydrolyze lipids and fat to glycerol and fatty acids. • **Trypsin and chymotrypsin:** They hydrolyse proteins to peptides. • **Nuclease:** They hydrolyse the nucleic acids to nucleotides.

Table 6. COMPONENTS WHICH PARTICIPATE IN THE DIGESTIVE PROCESS

5. Probiotics and prebiotics

The origin of probiotics can be found in Elie Metchnikoff's (1845-1961) view of the benefits of **fermentative bacteria**.

As it is known, the ingestion of food fermented in a lactic fermentation process is beneficial for the consumer's health so he proposed the implantation of these fermentative bacteria in the intestinal tract.

Later on, in agreement with Metchnikoff's theories, probiotics were said to be live microbial supplements which enhance the GI microbial balance improving the consumer's health.

According to the accepted definition of a probiotic, it should survive the digestive process in order to confer a health benefit and afterwards colonize the host's IECs. The interaction with the intestinal mucosa is considered to be important but not indispensable so as to provide the strain with probiotic properties.

Otherwise, the adhesion to the intestinal mucosa is not taken into account when referring a strain as a probiotic or not. Nevertheless, the referred adhesion to the intestinal mucosa has many benefits such as giving the strain a close position to the intestinal immune system and therefore, the possibility of modulating it.

Probiotics are capable of inhibiting and removing enteric pathogens* even though this capacity totally depends on each strain. This can be carried out by virtue of the production of inhibitory compounds, stimulation of the intestinal immune system and the competition with pathogens for adhesion to the IECs and for nutritional sources.

Nevertheless, some fermented foodstuff may be used as probiotics due to the concentration of beneficial bacteria they contain. Some examples can be yogurts, sauer kraut, kefir, pickles, miso, kombucha, kimchi and many others. **Fermented food** by LAB has been proven to be beneficial for health due to the fact that when being ingested, this type of bacteria arrive to the GIT offering health advantages.

Prebiotics are a **supplement** that is made of a dietary fiber and its use is to **nourish** the beneficial microorganisms that are already located in the GIT. Unlike probiotics that must arrive to the GIT alive and therefore they must resist low pH conditions, different temperatures and huge amounts of time; prebiotics are not affected by the aforementioned factors.

On the one hand, they can be obtained from the ingestion of specific natural products. For instance, the chicory root is believed to be the foodstuff with the highest percentage of prebiotic fiber per gram.

On the other hand, they can be acquired from the intake of prebiotic supplements on the market.

6. Antimicrobial drugs

Antibiotics are chemical substances which have the ability to destroy a big variety of microorganisms or to stop their reproduction. They can act on most bacteria, some fungi and protozoa. However, they must not affect the basic functions of the bacteria's host. They can be synthesized naturally by fungi or bacteria or in a laboratory.
There are two types of antimicrobial drugs:

- **Bactericidal**: This type of antibiotic kills microbes directly. Its action is based on inhibiting the growing of microbes or their multiplication. Its mechanism of action is based on interfering in the synthesis or union of peptidoglycan which is the main component of the cellular wall. They impede the joining of cellular wall elements to it. In that way, when the pressure inside cell rises, it beaks causing the bacterium to die.
- **Bacteriostatic**: On the other hand, this kind of antimicrobial drug does not kill microbes but stop their growth. This can be achieved in two different ways. The first one is stopping protein synthesis by virtue of inhibiting ribosome synthesis. The second one consists of inhibiting the DNA synthesis by blocking transcription or duplication.

Many antibiotics work by damaging the plasma membrane on account of the fact that it is vital for the cell. Mainly, they affect the bacterial wall, exposing the plasma membrane to being injured. But there are other compounds used as disinfectants such as alcohol, which directly affect the plasma membrane.
A few antimicrobial drugs inhibit the protein synthesis which takes place in the ribosomes. Eukaryotic ribosomes differ from the ones in prokaryotic cells due to their size and on account of this; the aforesaid antimicrobial agents only kill bacteria.

6.1. Resistance to antibiotics

Bacteria that have the ability to survive and grow in an environment with antibiotic substances which normally inhibit or kill bacteria are known to have antibiotic resistance. This aforesaid antibiotic resistance can be inherent or acquired.

Regarding to the inherent, its definition states that some bacterial strains are not susceptible to specific antibiotics. And therefore, they can survive and even grow in its presence without being inhibited or killed.

However, acquired resistance can be explained as a mutation of a gene which cannot be transferred and as a resistance gene which may have been transferred from a bacterium. See the transfer process in the **Nucleoid** section.

In the subsequent sections, there will be described some details about the antimicrobial drug Amoxicillin and its association with clavulanic acid. The information given will be of use in the7.4. Antibiogram section.

6.2. Amoxicillin

Amoxicillin is a **bactericidal** antibiotic which kills microbes directly. It acts inhibiting transpeptidation and provoking autolysis of the cell wall as many betalactamic antimicrobial drugs.

The spectrum of Amoxicillin is wide-ranged and it includes most Gram-positive bacteria and some anaerobic strains.

6.3. Clavulanic acid

Clavulanic acid is in charge of the inhibition of most **β-lactamases** clinically needed that have been proven to hydrolyze Amoxicillin.

6.4. Amoxicillin/clavulanic acid

The **association** of the antibiotic Amoxicillin with clavulanic action has been largely employed in medicine since its invention. It consists of the combination of Amoxicillin with the clavulanic acid which partially avoids the resistance of some bacteria to Amoxicillin. In that way, the action of the aforesaid antimicrobial drug is brought to bacterial strains which produce β-lactamases that is believed to be the cause of the antibiotic resistance.

Furthermore, with this association it can be achieved the introduction of some bacterial strains to the Amoxicillin spectrum.

In addition, it has been proven an imperceptible modification of the intestinal flora.

7. Laboratory experiences [1] [2]

7.1. Are bacteria in probiotics alive?

One of the objectives of the present work was to analyze whether bacteria contained in probiotics were alive or not. In order to achieve a conclusion regarding this, put some probiotics in an agar plate so as to check if bacteria in it grow or not.

The hypothesis thought about was that bacteria in probiotics were alive due to the fact that the ingestion of dead bacteria would not confer a health benefit at all. Still, it was contemplated the possibility that they were not alive because they must survive much time in the probiotic recipient in conditions that may not be favorable.

- **Equipment and material**

There were used two different types of probiotics: Ecoceutics: digestive system and Lactoflora which protects the intestinal lining.

The first one was made of three different bacterial strains: *L. gasseri*, *Bif. bifidum* and *Bif. longum*. It is an alimentary complement which is contained in capsules.

On the other hand, Lactoflora was made of four different bacterial strains: *Bif. lactis BI-04, L. acidophilus La-14, L. plantarum Lp-115* and *L. paracasei Lpc-37*. It was contained in single-dose bottles and it had expired in March 2017, which can make the results vary slightly.

Furthermore, the material used was the following: 10 ml graduated cylinder, griffin form beaker, a glass rod, two Petri dishes and laboratory oven.

- **Procedure**

1. Mix the Ecoceutics probiotic with 6ml of distilled water in a Griffin form beaker. Use distilled water so as not to affect bacteria with osmotic changes.
2. Stir until it is completely blended with the help of a glass rod.
3. Add 1 ml of the mixture to the agar plate.
4. Put 1 ml of the Lactoflora probiotics directly to another Petri dish because it is not needed to add water.
5. Put both agar plates in the laboratory oven at 35º and leave them for three days. After this period of time, not only would there be an evidence of bacterial growth but colonies would have also been formed.

[1] All the experiences, even if not stated, were repeated at least twice so as to confirm the veracity of the results.

[2] Each one of the photos showed in this section was taken by the author.

- **Results**

It could be checked that bacteria had definitely grown. Different types of bacterial colonies could be seen in each Petri dish. To identify each one of them, they were observed through the binocular loupe.

Identification of L. and Bif. should be carried out on a molecular approach. Nevertheless, in the present study, only bacterial colonies characteristics and their reaction to the Gram-stain method have been considered.

When observing the agar plate with the Ecoceutics probiotics under the binocular loupe; there could be recognized two different bacterial colonies and mildew with yellow dots, candidates of being the third bacterial colony.

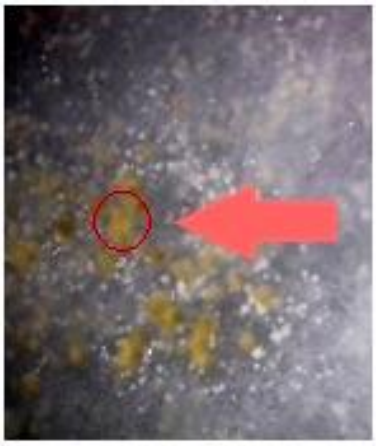


Figure 15

On the one hand, in this image it can be seen the referred mildow with several small yellow dots that could be bacterial colonies which tended to be punctiform. Their shape was circular with quite entire margins. Their colour was an intense, vivid and opaque yellow. They were convex colonies with a dull surface. With the help of the Gram-stain method, when looking at them under the microscope it will be checked whether they have a similar structure to bacteria or not.

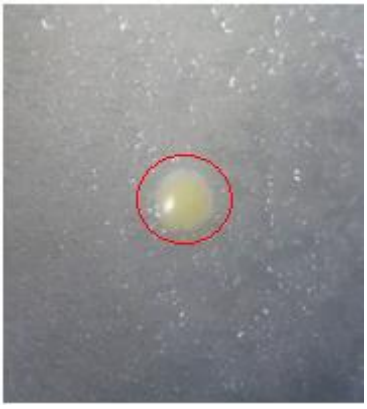


Figure 16

On the other hand, the second colony observed despite being also a yellow colony, it was very different from the one before. It was also circular with entire margins. Its surface was glistening accompanied by a viscous texture. However, the color was a pale and cloudy yellow which seemed a bit translucent. It was also a convex bacterial colony and there could be find approximately 6 of them on the whole agar plate.

Figure 17

Finally, the last colony observed was bigger than the two previous ones. It did not have a particular color and it was translucent. Its form was circular with a differentiated, white and filiform margin. Its texture was viscous and the surface was shimmering. However, the texture was not uniform considering that there seemed to be small and white dots on top of it.

However, when looking at the agar plate with the Lactoflora probiotics under the binocular loupe, despite of containing 4 different bacterial strains, only three different bacterial colonies were distinguished.

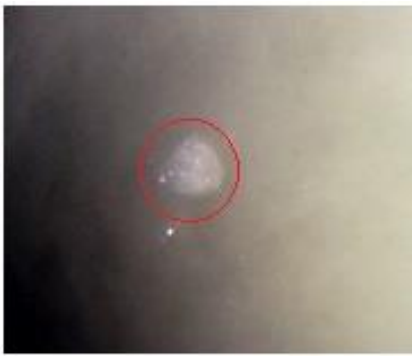


Figure 18

The colony which can be seen in this image was the most numerous one due to the fact that there were over 50 of them. Their form was not entirely circular but quite close to it. The margins were entire and the surface glistening. They had small, white and shimmering dots on its surface. They looked moist and viscous but firm. These raised colonies were slightly white and translucent.

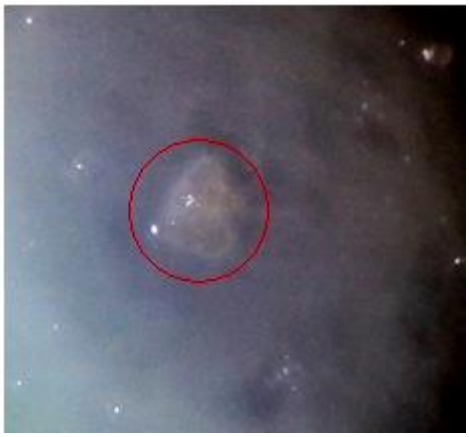


Figure 19

These colonies quite resemble the ones exposed previously but they differ in some aspects. Their similarities do not go further than a comparable texture and its corresponding white and shimmering dots. They differ in form, due to the fact that this one is further from being circular than the first one. Its margins are differentiated from the rest of the colony, being whiter and less translucent. Its color is slightly distinct, having a faintly yellowish and translucent hue. There were approximately five of them. They were bigger than the previous ones.

Figure 20

The last colony observed was completely different from the other ones. There were two of them and they had two differentiated parts. The first one was a colony that consisted of two circular ones united. They were transparent, convex and with entire margins. They seemed to be moist and viscous. They had a small colony attached. Its color was a shimmering, iridescent and opaque yellow. It was not totally circular and it had entire margins. It was a pulvinate colony with completely entire margins.

7.2. Gram stain method

Having realized that some bacteria had grown in each Petri dish, the next step was to identify which ones of them had actually arised.

The Gram stain method can help you to have more information about each type of bacteria and consequently, identifying them easier. Besides, more information about each type of bacteria would be compiled.

The Gram stain method is one of the most used staining methods in microbiology due to fact that it divides bacteria, depending on their cell wall structure, into two different groups: Gram-positive and Gram-negative bacteria. These two groups differ in other aspects as well as from their main difference which is the structure and organization of the cell wall.

When applying the reagents of the method, both groups react differently. Both become purple when the crystal violet dye is applied because it enters the cytoplasm of the cells. The iodine, when being applied, forms crystals with the crystal violet that are too big to escape from the inside of the cell.

In the case of the Gram-positive cells, the alcohol and acetone added dehydrate the murein making it more impermeable to the crystal violet and thus, it is retained on the inside.

On the other hand, the outer membrane in Gram-negative cells is dissolved and the peptidoglycan layer is perforated. As a consequence, the crystal violet-iodine is released. As a result, Gram-negative cells appear colorless under a microscopic view in that moment. Afterwards, they dye red when the Safranin dye is applied.

- **Equipment and material**

Acetone, Bunsen burner, cover slip, crystal violet, cultures, distilled water, dropper, ethanol 96º, glass slide, immersion oil, laboratory tweezers, Lugol's iodine, microscope, Safranin and a wire loop.

- **Procedure**

1. First of all, put a distilled water drop on the centre of the glass slide.
2. Then, transfer the bacterial colony to the glass slide using the wire loop that you have previously sterilized. To sterilize the wire loop place it above the flame of the Bunsen burner until it turns red. After that, wait a few minutes before transferring the colony so that the wire loop cools so as not to burn the bacteria. Whilst waiting, be careful not to touch anything with it and contaminating it.
3. When transferring the colony, smear it on the glass slide in order to form a thin film. In this way, the different bacteria will distribute over the glass slide and it will be easier to observe them under the microscope. It is important to use fresh colonies on account of the loss of their ability to retain the stain as they age.
4. Afterwards, fix the sample with heat. To do that, subject the glass slide with the laboratory tweezers and pass it slightly above the flame of the Bunsen burner. Be careful not to burn it.
5. Then, cover the fixed cells with crystal violet for 1 minute. To remove the excess of colorant, rinse with distilled water. Under a microscopic view, all cells appear to be purple at this point.
6. Later, Lugol's iodine which is used like mordant is added and it increases the affinity for the next dye. Rinse with distilled water again after 30 seconds. At this moment, all the cells still appear purple.
7. Next, decolorize the sample with a solution made of acetone and ethanol in equal parts. Add the aforementioned solution using the dropper and tilting the glass slide at an angle. Repeat the same process until the drop comes out colorless. Gram-positive bacteria will retain the crystal violet dye while Gram-negative bacteria will appear colorless under a microscope. Wash off the excess of ethanol and acetone with distilled water.

8. Dye the cells with the Safranin colorant for 1 minute. This will make the Gram-negative bacteria turn pink whilst it does not affect the Gram-positive bacteria which remain purple. Rinse with water and dry the glass slide carefully with filter paper.
9. Put a cover slip above the sample.
10. Observe the preparation under the microscope at different magnifications. When using the immersion magnification, put a drop of the immersion oil on the coverslip.

- **Results**

Subsequently, the different bacteria observed will be exposed, described and analyzed.

In this picture, there are shown the different samples used in the experiment. The 1st and 2nd one belong to the Ecoceutics probiotics. The first sample was taken from the colony that can be seen in figure 15 and the second one was selected from the colony shown in figure 16.

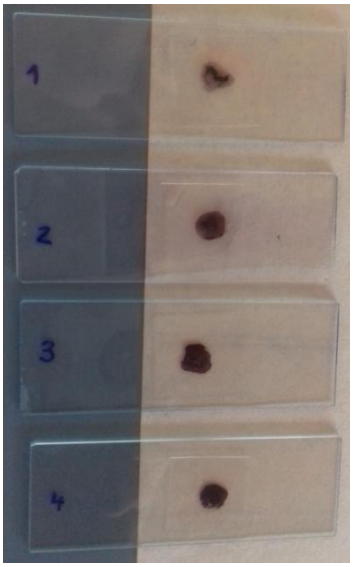


Figure 21

On the other hand, the 3rd and 4th samples belong to the Lactoflora probiotics. The third one was taken from the colony in figure 17 while the fourth one was taken from the colony observed in figure 18.

When the photo was taken, the samples had undergone half of the Gram stain method; that is why they are all purple-colored.

However, it can be seen the 3rd and 4th samples looked darker than the others belonging to the Ecoceutics. That is because they had not been properly smeared and thus they did not form a tiny film.

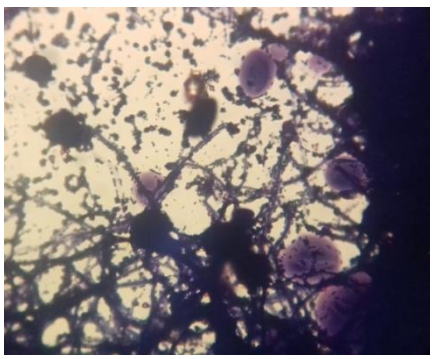


Figure 22

When observing them under the microscope, there could not be distinguished the different bacteria on account of the fact that when the bacterial colonies had been smeared with the help of the sterilized wire loop they had not been properly distributed.

In this image it can be seen the different bacteria gathered all together forming a purple network with some foreigner particles that might have been accidentally added.

In the first sample, there could only be observed Gram-positive bacteria which looked like coccobacilli and appeared individually. Owing to that, it could be the *L. gasseri*.

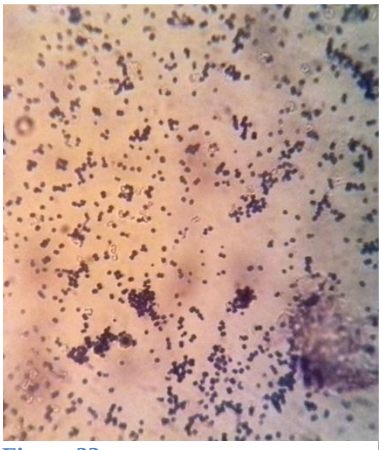


Figure 23

In the second sample, there could be distinguished three different types of bacteria that reacted in different ways to the Gram stain method.

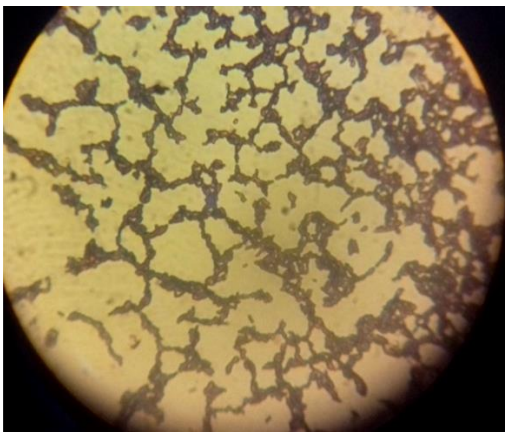


Figure 24

The first type of bacteria can be seen in figure 24 and as it is purple-colored, it can be known that it belongs to the group of Gram-positive bacteria. Furthermore, there were not any bacteria in the Ecoceutics probiotic which belongs to the Gram-positive group. Therefore, they might be a result of a contaminated sample.

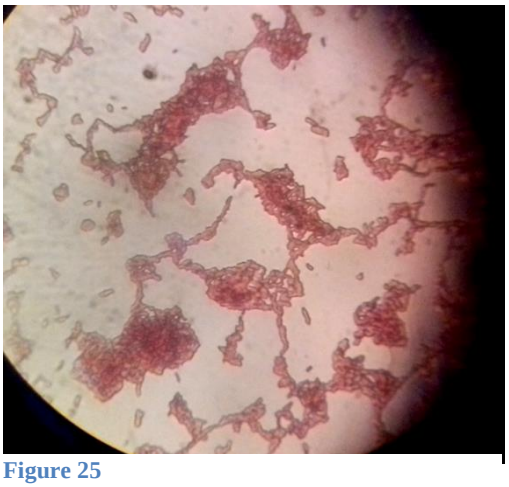


Figure 25

The second type of bacteria which could be differentiated was red-stained and therefore it belonged to the Gram-negative group. In this case, it could be the Bif. longum, the Bif. bifidum or L. gasseri.

However, there was a third type of bacteria which was not dyed by the Gram stain method. That means, as it was explained in the **Bacterial wall** section, that it must belong to the mollicutes or archaebacteria strains.

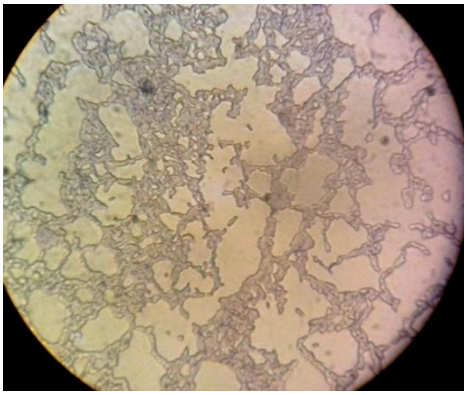


Figure 26

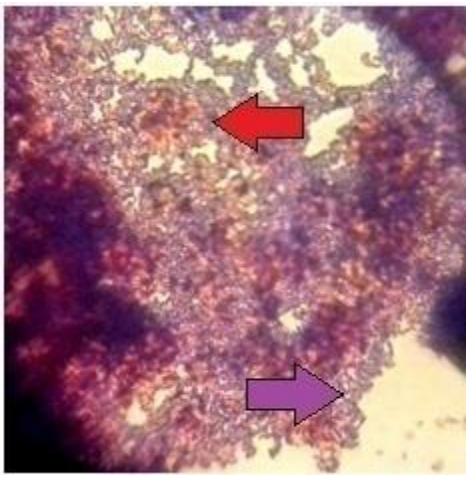


Figure 27

In the third sample, there could be observed two different bacterial strains that appeared all together. Bacterial colonies are formed with just one bacterial strain so this sample might have been taken from two distinct colonies accidentally.
There could be distinguished Gram-positive and Gram-negative bacteria which have been indicated with a purple and a red arrow respectively.

The Gram-positive ones could be L. acidophilus La-14, L. plantarum Lp-115, L. paracasei Lpc-37 or Bif. lactis due to the fact that they all stain purple in the Gram stain method. On the contrary, the bacteria which are red-colored could be bacteria from the air which have accidentally been introduced.

Finally, the bacteria observed in the last sample were Gram-positive and therefore it could be any of the bacterial strains contained in the Lactoflora probiotic. They appeared individually and in pairs and did not have a circular shape but an extended one.

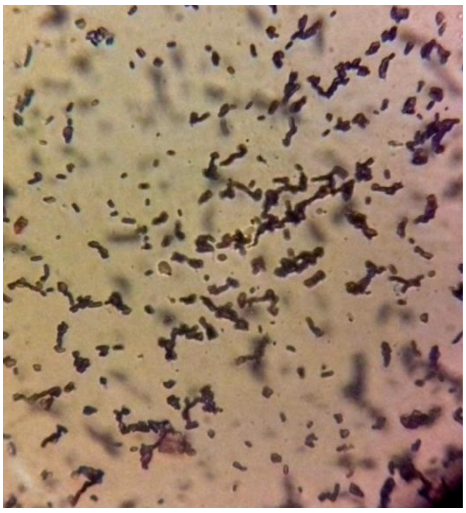


Figure28

7.3. Simulation of the digestive process

Once it was checked that bacteria in probiotics are alive, it was set out another problem: Do they survive the digestive process and therefore arrive alive to the GIT?

As the execution of the afore-said process requires complex material, it had to be adapted to the possibilities of the author.

The experience was divided into two separated procedures to facilitate the elaboration. This division enabled the separation of the processes which take place in the stomach and the ones carried out in the small intestine.

With a view of resembling the low pH conditions in the stomach, hydrochloric acid was used considering the same concentration in which it is found.

So as to reproduce the pancreatic juice, Pankreoflat tablets were used. The referred pills contain enzymes that can be found in the pancreatic juice such as amylase, protease and lipase.

On the one hand, in Gram-negative cells there is an external lipidic membrane which could be hydrolyzed by the enzyme lipase which is in charge of the hydrolysis of lipids and fats to glycerol and fatty acids. However, there are not any Gram-negative bacteria in any of the probiotics used.

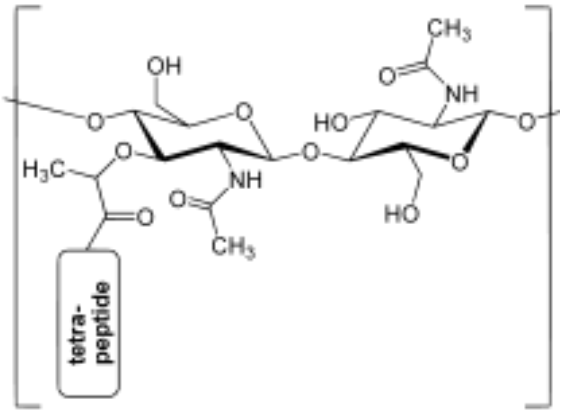


Figure 29. PEPTIDOGLYCAN

https://es.wikipedia.org/

Besides, amylase was thought to be able of maybe affecting the cells. On account of its action in which the glycosidic bond α(1→4) is broken. The referred glycosidic bond joins the glucose monosaccharide. This would affect both types of cell.

On the other hand, the protease enzyme is said to carry out the proteolysis by breaking the peptide bond. That is why it was thought that it could break the unions between the chains of amino acids in the peptidoglycan causing the cell breakdown and the subsequent death. Gram-negative and Gram-positive cells have peptidoglycan as the main component of their bacterial wall, so this could affect both of them.

However, regarding the hydrochloric acid, it was believed that bacteria would resist the low pH although the corrosive nature of it.

- **Material**

10 ml Graduated cylinder, griffin form beaker, Ecoceutics probiotics, hydrochloric acid, Pankreoflat, a glass rod, mortar and pestle.

- **Procedure**

PANCREATIC JUICE

1. Firstly, mix one capsule of the probiotics used with 12 ml of water in a beaker form.
2. In the second place, grind one pill of Pankreoflat with the mortar and the pestle. When it is completely reduced to powder, add it to the beaker form.
3. Next, stir with the help of a glass rod until it is totally blended.
4. Afterwards, put it in the laboratory oven at 36.5º for 3 hours, which is an approximation of the time that it would pass in the small intestine. The temperature of the laboratory oven simulates the human temperature which is around 36.5º.
   Cover the beaker form so as to simulate the effect of being in the intestine.
5. When the period of time has passed, sew 2ml of the mixture in an agar medium.
6. Put the aforementioned Petri dish in the laboratory oven at 35º for a minimum of 24 hours so as to check whether bacteria had survived or not. Turn the agar plate so that condensation of water does not interfere with the bacterial growth.
   After this period of time, bacteria may have started the formation of colonies. However, they would not be entirely formed.

GASTRIC JUICE

1. Add the content of a probiotic capsule to the griffin form beaker.
2. Next, prepare the dissolution of hydrochloric acid. The operations were the following so as to obtain 6ml of 0.082M from a 0.5M hydrochloric acid.

   The concentration of HCl presented in the stomach is the one stated before which was reproduced in the experiment.

$$6mL\ dissolution \cdot \frac{0.082\ HCl\ moles}{1000\ ml\ dissolution} \cdot \frac{1000\ ml\ HCl}{0.5\ HCl\ moles} = 0.984\ ml\ HCl$$
$$\approx 1\ mL\ HCl$$

3. Measure the referred quantity of HCl with a graduated cylinder and put it into a griffin form beaker.
4. Fill with water the 5 remaining ml. Proceed carefully when adding the water to the HCl because if not it can provoke burns.
5. Add the dissolution to the griffin form beaker containing the probiotic.
6. Afterwards, cover it with a filter paper so as to simulate the conditions in the stomach.
7. Put it in the laboratory oven for approximately 2 hours at 36.5º.
8. After the period of time has passed, sew 2 ml of the mixture in an agar plate. Leave it in the laboratory oven at 35º for 24 hours. Place the Petri dish face down to prevent the interference of water condensation with bacterial growth.
9. Check whether bacterial have grown or not and identify the different bacterial strains according to the characteristics.

- **Results**

PANCREATIC JUICE

The results obtained are shown in figure 30 and in figure 31.

As it was only left in the laboratory 24 hours, bacterial growth is evident but the colonies were not completely formed.

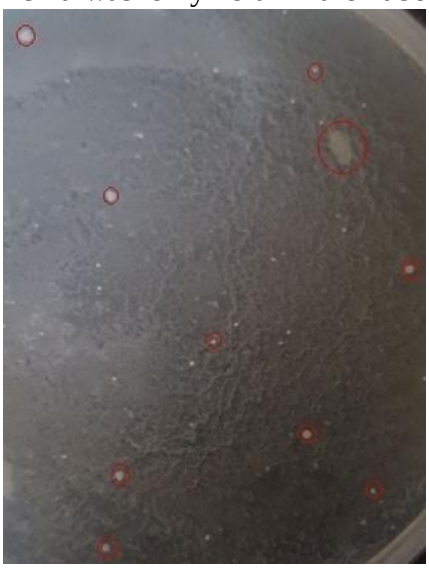


Figure 30

Nevertheless, there can be distinguished the three different bacterial strains that had started gathering together so as to form colonies.

First of all, the biggest colony found resembled the one seen in figure 15figure 15 and as it was said in the previous section, it could be L. gasseri.

However, there could be seen smaller colonies which could be comparable to the ones observed in figure 17 and in figure 16. They could be formed of Bif. Longum and Bif. Bifidum.

GASTRIC JUICE

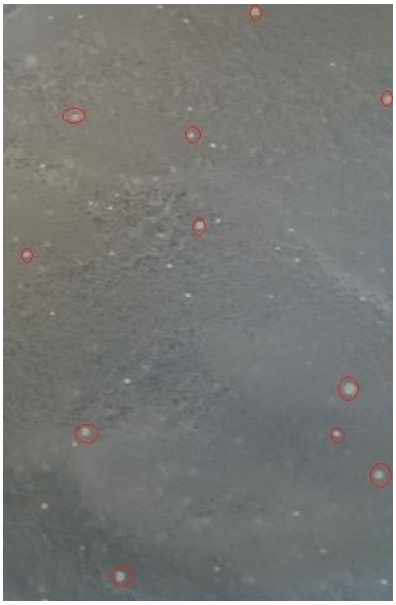


Figure 31

In this image it can be observed the bacterial growth after the experience with HCl. This indicates the survival of bacteria even though in presence of the simulated gastric acid.

There could be distinguished different types of colonies which correspond to the three types of bacteria contained in the probiotic used.

However, as it was left only 24 hours, colonies have just started forming.

This would indicate the survival of all the bacterial strains used for the experience.

This means that they might arrive alive to its destination where they are going to perform their beneficial action.

After having realized that bacteria in probiotics could resist the digestive process and arrive alive to its destination, the possibility that when they arrive to its destination some conditions could affect their living cycle was contemplated. These aforementioned conditions could be the intake of antibiotics or alcoholic drinks.

7.4. Antibiogram

In order to prove if antibiotics do affect the efficacy of probiotics I did an antibiogram to see if bacteria in the Ecovital probiotics were resistant to one of the most common antibiotics: Amoxicillin. I used two different types of antibiotics:

- Amoxicillin/clavulanic acid Cinfa 250/62,5 mg
- Amoxicillin Normon 250 mg/3 ml

The main difference between both of them is that the first one has another component: the clavulanic acid.

The clavulanic acid ($C_8H_9NO_5$) is used in combination with penicillin-group antibiotics as a result of the fact that it inhibits β-lactamases that are located in microorganisms which are resistant to penicillins. Otherwise, they would inactivate the function of the penicillin. That is to say that it overcomes the penicillin resistance which bacteria that secrete beta-lactamase enzymes have.

As Amoxicillin is a bactericidal antibiotic, it is supposed to kill some specific bacterial strains. All bacteria used for the experiment belong to the Gram-positive group having a thick bacterial wall. Owing to that, I believe that the antibiotic will have it more difficult interfering with the synthesis or union of peptidoglycan and therefore, I believe that they will not be affected.

- **Material**

10 ml graduated cylinder, Antibacterial drugs (Amoxicillin Normon 250 mg/3 ml and Amoxicillin/ clavulanic acid Cinfa 250/62,5 mg), bacterial culture (Ecovital), Bunsen burner, cotton swabs, ethanol 96º, filter paper, glass rod, griffin form beaker, hole puncher, laboratory tweezers, laboratory oven and the culture medium.

- **Procedure**

1. The first step is to sow the bacteria on the agar medium.First of all, open one capsule of the probiotics and add its content to an empty Griffin form beaker. With the help of a 10 ml graduated cylinder measure 6 ml of distilled water and add them to the Griffin form beaker. Stir with a glass rod until it is blended.
2. Using a cotton bud distribute the aforementioned mixture on the Mueller Hinton agar. Do this step placing the Mueller Hinton agar plate near the Bunsen burner flame in order not to contaminate it. The space around the flame is sterilized due to the high temperatures in which microorganisms cannot live.
3. Next, prepare the antimicrobial drugs for the experience. In the case that the antibiotics are in a powder format, mix them with water first.
4. In the instance of the Cinfa Amoxicillin/ clavulanic acid 250/ 62.5 mg, I had to calculate the concentration of water needed per gram of Amoxicillin, so as to put the same amount of Amoxicillin in both cases. In each envelope, there are 250 Amoxicillin mg and 62.5 clavulanic acid mg and that makes a total of 312.5 mg of powder.
5. Determine the quantity of powder needed in order to have 10 Amoxicillin mg through the following conversion factor.

$$10\ Amox.mg \cdot \frac{312.5\ powder\ mg}{250\ Amox.mg} = 12.5\ powder\ mg$$

Per 10 mg of Amoxicillin, add 5 ml of distilled water.

On the other hand, in the case of Normon Amoxicillin 250 mg/5 ml, mix 10 mg of Amoxicillin with 5 ml of distilled water. Measure the quantity of Amoxicillin with the help of a top-loading balance.

6. Next, drench the pieces of filter paper in the antibiotic, holding them with the laboratory tweezers which have been previously sterilized. To sterilize them, soak them in ethanol and afterwards pass them above the flame of the Bunsen burner. In case of not having rounded pieces of filter paper, do them with the help of a hole puncher. When placing the pieces of filter paper impregnated with the antibiotic on the Mueller Hinton agar, press them slightly in order to fix them.
7. Turn around the Mueller Hinton agar plate so the water drops on the surface do not affect the growth of the bacteria and put it in the laboratory oven from 18 to 24 hours.
8. After this period of time, take the agar plate of the oven and observe the growth of the bacterial colonies.
9. Then, measure the diameter of the surface on which bacteria have not grown. Depending on how big it is, they are more or less sensitive to the antibiotic. If bacteria continue their growth bordering the piece of filter paper it means that the antibiotic does not affect them and therefore they have resistance to it.

- **Results**

The first time doing the antibiogram a result was not achieved. Next, it will be shown the different reasons why it may have failed owing to the fact that the procedure it was used differs in some aspects from the one used by scientists.

First of all, normally it is used a specific type of agar called Mueller Hinton agar while in my case it was used thestandardPetri dish.

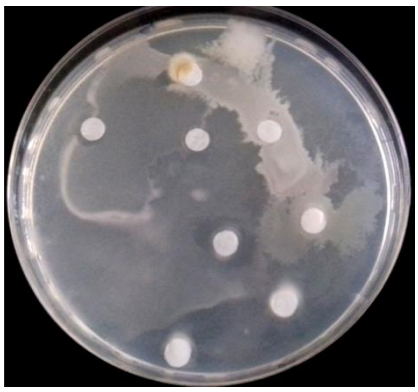


Figure 32

Secondly, some cultures are taken to the agar plate forming just a thin film whilst I mixed the content in an Ecoceutic capsule with 1 ml of distilled water and added it to the Petri dish plate.

Finally, it should be left in the laboratory oven from 18 to 24 hours as a maximum whereas it was left three days in the laboratory oven on account of the fact that in the previous experiments it had also been left for the same period of time.

The fact of adding an undue quantity of bacteria combined with the huge amount of time it was left in the laboratory oven are probably the reasons of the excessive bacterial growth that can be seen in figure 32.

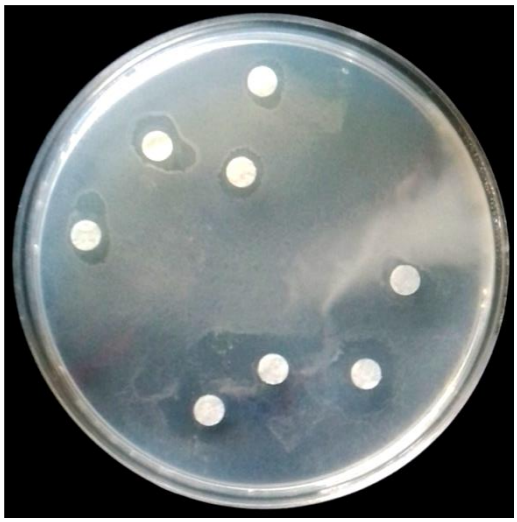


Figure 33

The antibiogram was realized again, changing the possible factors that may have caused the antibiogram to go wrong the previous time. The factors changed were:

- The huge amount of time that had passed until it was removed from the laboratory oven going from 3 days to 24 hours. Which is actually the maximum recommended.
- Adding less quantity of bacteria. In this occasion, a capsule of probiotics was mixed with 6 ml of distilled water as always but only 2 ml of this mixture was added to the agar plate.

This occasion, measurable inhibition halos were achieved. The four pieces of filter paper observed in the upper part correspond with the association of Amoxicillin and clavulanic acid. On the other hand, the ones located in the lower region resemble the ones with only Amoxicillin. Its inhibition halus was not as marked as the one belonging to the association. This could be a result of the bacteria in probiotics being able to resist Amoxicillin by secreting β-lactamases. This resistance could have been acquired thanks to a spontaneous mutation which is beneficial for these bacteria.

7.5. Does alcohol consumption affect the action of probiotics?

As it has been exposed previously, alcohol does not reach further than the small intestine due to the fact that ADH and CYP2E1 enzymes degrade alcohol to acetic acid. This transformation happens in the duodenum, where the bile is released from the liver to the small intestine. From the duodenum alcohol is found as acetaldehyde and later as acetic acid until it is metabolized.

In the present experiment it will be checked whether alcohol consumption does affect bacterial growth in the upper part of the GIT. The microbiota part of the small intestine found previously than the duodenum will be simulated using the Ecoceutics probiotics which have been employed in the previews experiments. One of the bacterial strains contained in it was used in a study carried out by Mainville, Arcand and Farnworth in the year 2005 in order to simulate the upper part of the GIT. The referred bacterial strain is Bif. longum.

- **Material**

The material used for the experiment is the following: laboratory tweezers, glass rod, 10 ml glass beaker, 10 ml graduated cyinder, distilled water, filter paper, hole puncher, petri dish, Ecoceutics probiotics and alcoholic drinks. In the present experience, the alcoholic beverages utilized were the succeeding: Ginebra (43º), Bailey's (40º), ron (37.5º) and wine (11.5º).

- **Procedure**

The methodology used is very similar to the one employed in the antibiogram realization. Consequently, it will be briefly exposed.

1. Mix one capsule of the Ecoceutics probiotics with 6 ml of distilled water and after that, add 2 ml of this mixture to the agar medium. Move gently the petri dish in order to distribute bacteria over the whole surface. Do this step in the sterilized area near the flame of the Bunsen burner.
2. Drench the pieces of filter paper with alcohol using the laboratory tweezers. Before each use they must be sterilized by placing them above the flame of the Bunsen burner. In order to have more than one replica drench two pieces of filter paper in each drink. In case of not having pieces of filter paper, do them with the help of a hole puncher.

3. Mark on the inferior cover of the Petri dish with a permanent marker where will be the pieces of paper drenched in each drink placed.
4. Put the pieces of filter paper on the agar medium according to the marks previously done. Press slighlty so as to fix them. As stated before, effectuate this in the sterilized area near the Bunsen burner flame.
5. Place the Petri dish in the laboratory oven at 35ºC for a minimum of 18 and a maximum of 24 hours.

- **Results**

When looking at the Petri dish plate there cannot be seen any inhibition halos around the pieces of filter paper. On the contrary, it could be observed a uniform bacterial growth on the surface of the whole plate.

Figure34

Therefore, the conclusion extracted from this experience is that the bacterial strains used are not susceptible to the intake of alcoholic drinks.

That means that the intake of alcohol does not interfere with the action of probiotics.

Furthermore, it could also be concluded that bacterial strains in the upper GIT may not be affected by alcohol consumption due to the fact that in the experience its autochthonous microbiota was attempted to be resembled. Nevertheless, that cannot be affirmed with certainty owing to the fact that only one of the bacterial strains used is presented in the microbiota of the studied region. Accordingly, it can be concluded that the antibiotic intake does not affect the action of the utilized probiotic and besides, it does not affect the growth of Bif. Longum; a bacterial strain found in the upper GIT.

8. Conclusions

The microbiota has been proven to be really important for health by some recent studies. Owing to the fact that alterations in it can cause many illnesses which have been exposed throughout the present work.

As it is considerably hard to study the microbiota because it is truly difficult to obtain representative samples; it was thought that it could be studied from the point of view of probiotics which are believed to improve its health. Therefore, the main objectives of the work were to demonstrate whether probiotics are efficient and the importance of the microbiota.

As the most accepted definition of what a probiotic is, states that they contain live bacteria; in the first laboratory experience it was checked. It was analyzed the fact that they had to spend many days in a closed capsule which could result into the death of them. After carrying up the sewing of different probiotics in an agar plate, it was confirmed three days later the growth of bacterial colonies. With the help of the Gram-stain method, the bacteria which had actually grown were recognized; although it should have been carried out on a molecular approach for more accurate results.

After verifying the fact that they are alive in the referred capsules it was set up the problem of their survival or not to the digestive process so as to arrive alive to their destination and confer a health benefit. The process was simplified with the use of hydrochloric acid as a simulation of the gastric juice and Pankreoflat tablets which contain the enzymes in the pancreatic juice. On the one hand, the enzyme protease was believed to be capable of hydrolyzing the peptide bonds which join together the chains of peptidoglycan, causing the cell breakdown. The other enzymes were not considered to be harmful for bacteria. On the other hand, the hydrochloric acid was considered to be detrimental for bacteria due to its corrosive nature. However, it was proved their survival. The explanation given to it is that the enzyme may not be able to break the majority of the peptide bonds in each cell which did not cause its death. Otherwise, these bacteria could be tolerant to acid due to some mechanisms such as the neutralization of acid by the generation of regulators or specific compounds.

Once it has been confirmed the fact that they arrive alive to their destination it was set out the doubt of other factors which could affect them. The afore-said factors were the

intake of antibiotics and alcohol consumption. The antibiotic used for the experience was Amoxicillin which is bactericidal and therefore, it is believed to directly kill some bacterial strains. All bacteria used for the experiment have a thick bacterial wall according to their belonging to the Gram-positive group. In that way, it was believed that the antibiotic would not affect them. Actually, they were proven to be susceptible. This could be explained thanks to the composition of their bacterial wall which is basically made up only of peptidoglycan and consequently, when the unions are broken, the cell is destroyed. Referring to the alcohol, it was not proven any effect on the probiotics which may be a result of a mutation of the bacterial strains present in the microbiota.

However, the results obtained cannot be representative due to the fact that some errors may have been made. For instance, in the Gram stain method; there were cells which turned out to be Gram-negative although there were not any in the probiotics used. Possible explications are given to such errors. The only way to avoid them is repeating the experiences more than twice, having sterilized material and installations and more time to study all the possibilities.

To conclude, basing on the results achieved in the experiences, it could be said that the probiotics used were not proven to be affected by the digestive process nor by alcohol. However, they were susceptible to Amoxicillin. It must be point out the fact that even the experiences were repeated at least twice, the results cannot be assured. Furthermore, the conclusions are only valid for the probiotics used in the experiences.

Nevertheless, it could not be studied if the intake of probiotic tablets is essential for the microbiota health because their effects once they arrive could not be investigated. Furthermore, thanks to the theoretical research the importance of the microbiota and its functions could be assimilated.

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Glossary

- Catalase-negative: It is a group of bacteria differentiated from catalase-positive by means of the catalase test. Catalase is an enzyme which is produced by bacteria that breathe oxygen. On the contrary, bacteria which are anaerobes and as a consequence do not need oxygen for their respiratory processes, do not have the aforementioned enzyme.
- Chemo-organotroph: It is a type of organism which carries a process named chemotaxis so as to gain energy. It is a term which designs a process in which bacteria respond to a chemical substance to which they may be attracted or not.
- Coccobacilli: It refers to a bacterial shape which is between a coccus and a bacillus.
- Enteric pathogens: They are pathogenic bacteria which are normally located in the small or large intestine.
- Exogenous gene: This term is used for any DNA fragment which is found outside of the referred organism.