Universidade de Lisboa

Faculdade de Farmácia

Bacteriófagos e a Microbiota Intestinal

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Monografia orientada pela Professora Doutora Madalena Maria Vilela Pimentel, Professora Associada com Agregação

Mestrado Integrado em Ciências Farmacêuticas

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Resumo

O corpo humano constitui um habitat para uma multitude de microrganismos, nomeadamente bactérias, vírus e fungos, entre outros, no seu conjunto designado por microbiota humana. Os bacteriófagos, vírus que infetam bactérias, representam a maior porção da microbiota humana e são particularmente importantes no intestino onde, em conjunto com bactérias, desempenham variados papéis, alguns benéficos para o hospedeiro humano. No intestino humano encontram-se principalmente bacteriófagos pertencentes à ordem *Caudovirales*, composta pelas famílias *Myoviridae*, *Siphoviridae* e *Podoviridae*. Também no intestino, os filos bacterianos predominantes incluem Firmicutes e Bacteroidetes, que englobam os géneros *Lactobacillus*, *Bacillus, Clostridioides*, *Enterococcus*, *Ruminococcus*, *Bacteroides* e *Prevotella*. Um desequilíbrio destas entidades, quer em relação uma a outra quer fora dos seus parâmetros normais (por exemplo, diminuição da população bacteriana devido a uma terapia antibiótica prolongada e descontrolada), resulta em disbiose. A disbiose intestinal prolongada pode estar envolvida em condições patológicas, das quais as mais relevantes são: (i) *diabetes mellitus* tipo 1 e tipo 2; (ii) Doença Inflamatória Intestinal; (iii) Doença de Crohn; (iv) Colite Ulcerosa; (v) Infeção por *Clostridioides difficile*; e (vi) cancro colorretal.

Nos últimos anos, os microrganismos intestinais ganharam grande relevância devido à sua capacidade de regular o sistema nervoso central, numa via denominada Eixo Intestino-Microbiota-Cérebro. Através de mecanismos diretos e indiretos, bacteriófagos e bactérias intestinais podem modelar positivamente funções cerebrais e até alterar o curso de algumas patologias neurodegenerativas, nomeadamente doença de Alzheimer, doença de Parkinson e perturbações do espectro do autismo. O aumento da investigação neste campo tem reforçado a teoria de que, para além de estarem envolvidos no desenvolvimento de doenças, os bacteriófagos também podem atuar como instrumentos para melhorar a saúde humana.

Embora nos últimos anos o conhecimento sobre bacteriófagos, especialmente os bacteriófagos intestinais, tenha vindo a aumentar, ainda há muito a saber sobre o seu papel exato na saúde humana e ainda é necessária investigação que permita apoiar a sua validade como estratégias terapêuticas.

Palavras-chave: bacteriófagos; microbiota intestinal humano; disbiose gastrointestinal; terapia fágica;

Abstract

The human body comprises a habitat for a plethora of microorganisms, referred to as Human microbiota, which comprises bacteria, viruses, fungi, and other microorganisms. Bacteriophages, viruses that infect bacteria, represent the largest portion of the human microbiota and are particularly important in the human gut where, along with bacteria, carry out several roles which can be beneficial to the human host. The predominant bacteriophages in the human gut belong to the order *Caudovirales*, comprising the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families. Also in the gut, the most predominant bacterial phyla include Firmicutes and Bacteroidetes, encompassing the *Lactobacillus*, *Bacillus*, *Clostridioides*, *Enterococcus*, *Ruminococcus*, *Bacteroides*, and *Prevotella* genera. An imbalance in these entities, either in relation to oneanother or outside their normal parameters (for example, decrease of the bacterial population due to prolonged and uncontrolled antibiotic therapy), results in dysbiosis. Prolonged gut dysbiosis can be involved in pathological conditions, from which the most relevant are: (i) type-1 and type-2 *diabetes mellitus*; (ii) Inflammatory Bowel Disease; (iii) Crohn's Disease; (iv) Ulcerative Colitis; (v) *Clostridioides difficile* infection; and (vi) colorectal cancer.

In recent years, gut microbes have garnered great relevance due to their ability to regulate the central nervous system, in a pathway denominated Gut-Microbiota-Brain Axis. Through both direct and indirect mechanisms bacteriophages and bacteria can positively alter brain functions and even change the course of some neurodegenerative diseases, namely Alzheimer's Disease, Parkinson's Disease and Autism Spectrum Disorder. Increasing research on this field further supports the theory that, besides being involved in the development of diseases, bacteriophages can also act as tools for improving human health.

Although in recent years, knowledge in the field of bacteriophages, especially gut bacteriophages, has been increasing, there is still much to be known about their exact role in human health and more studies need be outlined to support therapeutic validity.

Keywords: bacteriophages; human gut microbiota; gut dysbiosis; phage therapy;

Agradecimentos

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Aos meus amigos Eva, Inês, Isabel, Mariana e Pedro, um obrigado imensurável. Sem vocês estes últimos 5 anos teriam sido astronomicamente mais difíceis.

Por fim, aos meus queridos pais, avós, irmãos e cunhadas, o maior obrigado pelo apoio incansável que me deram, não só durante a realização desta monografia, não só durante os 5 anos que forma este curso, mas durante toda a vida.

Sem qualquer um de vós não estaria aqui, a concluir esta etapa.

Abreviaturas

AIEC – Adherent Invasive Escherichia coli

ANS – autonomic nervous system

ASD – autism spectrum disorder

BAM – Bacterial Adherence to Mucus

Cas – CRISPR associated protein

CD – Crohn's Disease

CDI – Clostridioides difficile infection

CRC – Colorectal cancer

CRISPR – clustered regularly interspaced short palindromic repeats

DNA – desoxyribonucleic acid

ds – double-stranded

EAEC – Enteroaggregative Escherichia coli

GABA – gamma-aminobutyric acid

IBD – Inflammatory Bowel Disease

KWD – kill-the-winner dynamics

LPS – lipopolysaccharides

PWD – piggyback-the-winner dynamics

RNA – ribonucleic acid

ROS – reactive oxygen species

RQD – Red Queen dynamics

SCFA – short-chain fatty acids

ss – single-stranded

T1DM – Type-1 *diabetes mellitus*

T2DM – Type-2 *diabetes mellitus*

TLR 2 – Toll-like receptor 2

TLR 9 – Toll-like receptor 9

UC – Ulcerative Colitis

VN – vagus nerve

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1. Introduction

Besides eukaryotic cells, the human body also constitutes a reservoir of various microorganisms, namely bacteria, viruses, fungi, and other microorganisms, also referred to as the human microbiota. The most studied component of this microbiota are bacteria, despite not being the most abundant entity within this group. In any ecosystem bacteria exist, bacteriophages are also present, in much larger numbers.

Bacteriophages, also known as phages, are obligatory parasites of bacteria. They were first discovered over a century ago, due to their ability to kill bacteria, offering a new approach to the treatment of bacterial infections. However, with the discovery of antibiotics and their astronomical rise as the front-line molecules to treat bacterial infections, bacteriophages took a step back. Presently, due to the development of antibiotic-resistant strains of bacteria (a distressful public health problem), bacteriophage study and phage therapy have gained a second spotlight for their ability to kill specific strains of bacteria, while leaving the commensal microbiota unharmed, a major side-effect of antibiotics. Moreover, even in cases of resistance development, phage have the ability to evolve side-by-side with bacteria, finding new mechanisms of infection, maintaining treatment efficacy.

Despite the overall ubiquity of the microbiota in the human body, the gastrointestinal tract has been one of the most studied systems, for its specific microbiota and abundance in these microbes. Currently, it is well established that the gut microbiome can influence and be influenced, by the overall health status of the body. In fact, recent research has demonstrated the impact of the gut microbiome in controlling the central nervous systems (gut-microbiota-brain axis), possibly affecting the development of neurodegenerative diseases. Being bacteriophages one of the central elements of the gut microbiota, these entities also play an important role in human health, through both direct and indirect processes. These mechanisms include, for example, modulating bacterial population and its behaviour through temperate and lytic life cycles, and directly stimulating host immunity.

2. Objectives

Considering the crucial role bacteriophages play in human health, specifically in the gut, the main objectives of this dissertation were to: (i) collect current data on the most significant and abundant bacteriophage families that inhabit the human gastrointestinal tract; (ii) review the delicate balance maintained in the gut microbiota and some of the conditions that might disrupt it, giving rise to pathological states; (iii) identify the relationship between bacteriophages imbalance and disease ; and (iv) present new forms of therapy, using bacteriophages, that are being investigated, particularly to treat gut associated infections.

3. Materials and Methods

The data base from which the research was made consisted of PubMed, ScienceDirect and Elsevier. All the articles analysed were written in English.

Special attention was given to more recent review articles and clinical studies, namely between the years 2020 and 2022. In an initial search, the keywords used to find these sources were “bacteriophages”, “microbiota; “human gut dysbiosis”, and “bacteriophages in the gut”. In a second stage, to narrow the search to more specific subjects, the keywords “bacteriophages in Inflammatory Bowel Disease”, “bacteriophages in Ulcerative Colitis”, “bacteriophages in Crohn’s Diseases”, “Gut-Microbiota-Brain Axis”, “bacteriophages and neurodegenerative diseases”, and “phage-therapy” were applied. Studies concerning new therapeutic approaches using bacteriophages were also analysed, with particular attention given to more recent studies.

The research period spanned between October 2021 and August 2022.

4. Bacteriophages

4.1. What Are They?

The discovery of bacteriophages can be simultaneously awarded to two researchers. Fredrick William Twort, in England circa 1915, and Félix d'Herelle, in France circa 1917 (1–3).

Bacteriophages (or phages) are viruses that can only infect and replicate in bacterial cells. They can be found in every ecosystem on earth, in a multitude of habitats, ranging from the oceans to the human gut, and are currently considered the most abundant biological entity worldwide, reaching up to 10^{31} particles. Generally, bacteriophages outnumber bacteria by a ratio of approximately ten to one (4). Bacteriophages are also present in the human body, being part of the microbiota, and when compared to eukaryotic viruses (such as *Herpesvirus*), they are present in much greater numbers. Bacteriophages account for approximately 90% of the human virome (5).

There are countless types of phages, all differing in size, morphology, and even type of genome. To this day, with the advances in technology, especially in the field of viral molecular genetics, the scientific community continues to see exponential growth in the discovery of new phages (6) Despite some genetic differences, all these phages share some degree of conservation when it comes to structural proteins. The genetic differences and the marked diversity of bacteriophages are believed to be originated from the environment in which the phage is included, by great selective pressures, due to presence and interaction with bacteria (6).

The bacteriophage-bacteria interaction is considered one of the most specific forms of interaction between living organisms. A phage may infect multiple strains of a single species or even be specific for a single bacterial strain, however, a few may be polyvalent, being able to infect more than one species, usually closely related. Bacteriophages are capable of searching the environment until they find a susceptible host to unleash the series of events that lead to infection and replication (7).

4.2. Bacteriophage Structure

Bacteriophages have various morphologies and sizes and can be made up of different components (3). Despite this, all bacteriophages present a capsid of proteinic nature, whose prime goal is to store the viral genome. When it comes to nucleic acids, bacteriophages can

either have a desoxyribonucleic acid (DNA) or a ribonucleic acid (RNA). Both these genomes can be arranged in double-stranded (ds) or single-stranded (ss) helixes (3,6,7). In addition to the genome and the capsid, phages can present other structures such as tails, connector complexes, and adsorption apparatus, to list a few (3,6,7). Tails are tubular proteinic structures responsible for forming a channel through which the phage genome passes into the host's cytoplasm. Connecting the capsid and the tail, one can find the connector complex (also called collar or neck). Lastly, the adsorption apparatus is responsible for establishing the bacteriophage-host connection (with high selectivity). Some of the structures mentioned can be better observed in Figure 1. Interestingly, to aid in the infection process, some bacteriophages also possess what are called virion-associated lysins which, depending on their type, can degrade the peptidoglycan in specific bonds (7,8).

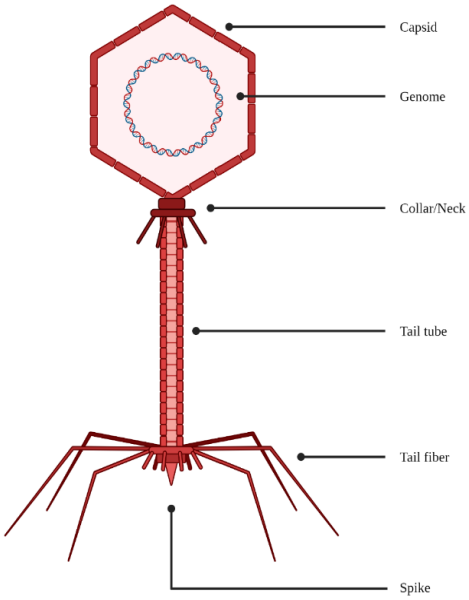

Figure 1. Structure of a common Myoviridae bacteriophage (Phage T4).
Created with BioRender.com

4.3. Bacteriophage Classification

The most used bacteriophage classification system takes into account the viral particle's morphology and type of nucleic acid. However, with the current advancements in bacteriophage genomics, a new classification system is being proposed, based on genome sequencing (7). When it comes to morphology, these classification methods refer to capsid morphology, in specific. This way, bacteriophages can be separated into four different groups: (i) tailed phages;

(ii) polyhedral phages; (iii) filamentous phages; and (iv) pleomorphic phages (7,9–11). Tailed phages are grouped in the order *Caudovirales*. Within this order, there are three different phage families, the *Myoviridae*, *Siphoviridae* and *Podoviridae*. All tailed phages (as their name implies) have dsDNA in a proteinic capsid, attached to a helical tail. Depending on the family of phages one is talking about, it's possible to distinguish some particularities of the tails of such phages. *Siphoviridae* phages have long (flexible or rigid) tails, while *Podoviridae* phages have short tails. *Myoviridae* phage's tails are contractile. Polyhedral phages are a group of bacteriophages that possess capsids with cubic symmetries. This group includes phages from four different families, including *Microviridae*, *Corticoviridae*, *Tectiviridae*, and *Cystoviridae*. Polyhedral phages can either have DNA or RNA genomes. *Tectiviridae* phages possess an icosahedral protein capsid that surrounds a lipid-containing vesicle. This vesicle has the special property of being able to change its configuration, forming a tail-like tube, for the phage to infect its host. Filamentous phages comprise the *Inoviridae* family and can either be long or short in length. Lastly, pleomorphic phages also only encompass one family, the *Plasmaviridae* . These phages don't possess a capsid, having only a "naked" dsDNA and lipoprotein envelopes. Pleomorphic phages infect only Mycoplasmas, are round in shape and are released from their hosts through budding (7,9–11).

4.4. Life Cycle and Replication

For a successful infection, every bacteriophage needs a susceptible host. One that expresses the right cell receptors to be recognized by the adsorption apparatus (2). According to their replication strategies, bacteriophages can be divided into two major groups, temperate and virulent or strictly lytic phages (10). Both infections processes start with the binding of a bacteriophage to a specific receptor at the bacterial surface (Figure 2-1). Shortly after, genome insertion into the bacterial cytoplasm occurs (Figure 2-2). Once in the cytoplasm, virulent phages can only proceed with the lytic cycle, replicating their genome, synthesising phage particles components, and assembling entire new phage-particles (Figure 2-3a). After this assembly, virulent phages lyse their hosts, which allows the release of new virions into the environment (Figure 2-4a), readily available to infect new hosts. On the other hand, temperate bacteriophages may follow a lytic or lysogenic cycle. In the latter, the phage DNA can be integrated in the bacterial genome (Figure 2-3b), in the form of a prophage (or independent circular replicon), replicating as the bacterium divides (in a phenomenon called passive

replication) (Figure 2-4b), for undefined periods of time. This way, every host descendent has a replica of the phage genome with itself, until an external stimulus such as radiation, lack of nutrients, temperature changes, chemicals, and antibiotics such as quinolones (which can cause DNA breaks) to mention a few, induces genome excision (Figure 2-5), phage replication, synthesis of new viral particles, and cell lysis. (2,7,10,12,13).

Interestingly, some filamentous bacteriophages such as the M13 phage do not lyse the host cell after replication. They can release active infectious viruses from their host without ever lysing the original cell. This process is achieved by establishing chronic infections in which the bacterial cell is continuously extruding infectious virions. This mechanism can also be called a non-lethal infection cycle (10,14).

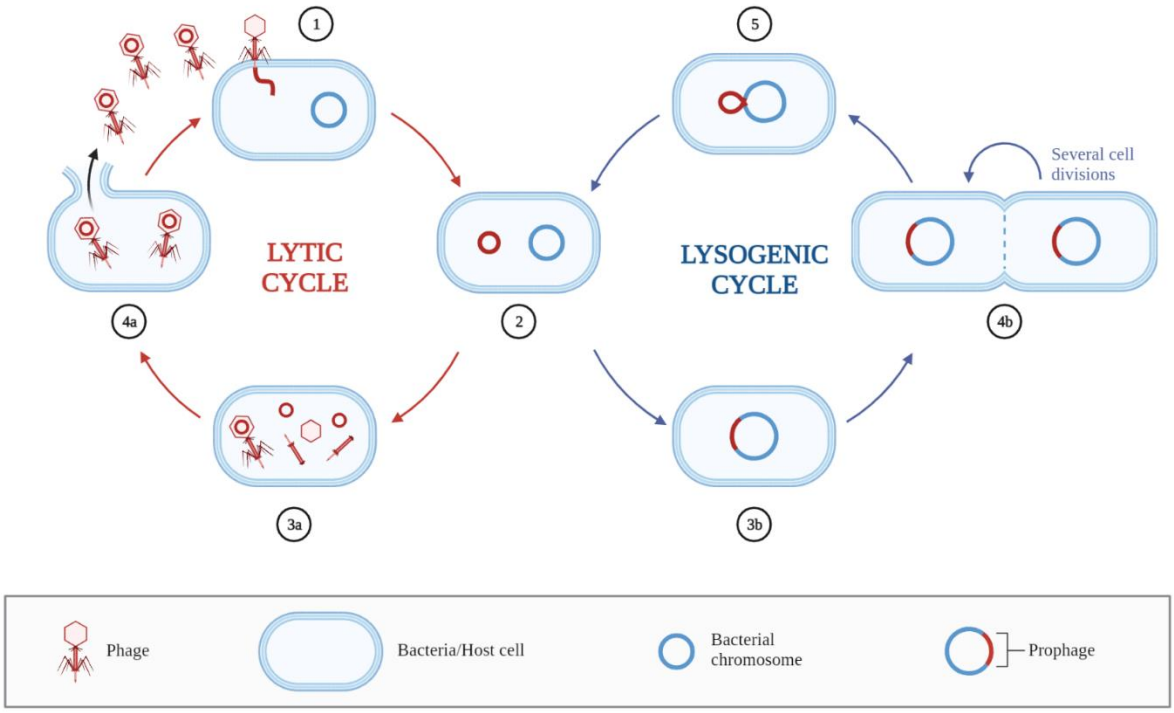

Figure 2. Stages of bacteriophage replication (lytic and lysogenic life cycles). 1 – Phage attachment to host cell through specific receptors at the surface; 2 – Phage DNA circularization; 3a – Synthesis of new phage particles; 4a – Lysis of host cell and release of new infectious phage particles into the environment; 3b – Integration of phage DNA into the bacterial genome; 4b – Normal replication of now lysogenic bacterium; 5 – Excision of phage genome due to external stimulus. Adapted from "Lytic and Lysogenic Cycle", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

4.5. Role of Bacteriophages on the Microbiota

Bacteriophages, in nature, play many important roles, the main being genetic transfer between bacteria and modulation of bacterial behaviour. Gene transfer, involving bacteriophages, also called transduction, can be further divided into "generalized" and "specialized" transduction. In "generalized" transduction, during phage particle assembly, random pieces of bacterial genome are mistakenly packed into the phage capsid, forming a new particle with fragments of bacterial genome inside it. Once released into the environment, this particle will inject the genome it's carrying into a susceptible host, which can recombine with the new host's DNA, creating a new bacterial variant. This type of genetic modulation is one of the main contributors to the high genetic variability in bacteria, as any part of the bacterial genome, as long as it has an appropriate size, can be transduced. On the other hand, in "specialized" transduction, mediated by temperate phages, only bacterial DNA segments adjacent to the integrated phage genome region can be incorporated in a new viral particle, as a result of an improper prophage excision (15).

As a direct consequence of genetic transfer between bacteria and also through lysogenic conversion, bacteria may acquire advantageous properties which can increase their survival fitness and result in a pathogenic lysogen. There are several examples of bacterial pathogens for which an increase in pathogenicity is associated with the secretion of phage encoded toxins. Some examples of lysogenic modifications are listed in Table 1, as well as the bacteria and bacteriophages involved. In a study conducted by Veses-Garcia et al. 2015, it was shown that bacteriophages such as phage $\phi 24_B$, carrying the Shiga toxin, can enhance gastric-acid resistance to Enterohemorrhagic *E. coli* strains, by increasing the expression of glutamic acid descarboxylase. This mechanism allows for the continuous exchange of protons with the environment, rendering the bacterium tolerant to high acidity levels (16). Bacteriophages can also carry other virulence factors having important roles in bacterial adhesion, invasion, and colonisation. Two examples are: (i) phages encoding the ankyrin protein gene that can hinder endothelial innate immune defences against *E. coli* species, leading to the expansion of pathogenic strains; and (ii) phages coding for adenosine-diphosphate-ribosyltransferases that can augment *Clostridioides difficile* colonisation in the mucosa by increasing its adherence capabilities (5).

On the other hand, bacterial population modulation is achieved through something mentioned earlier, specific predation of bacteriophages. By targeting a specific group of

bacteria, and infecting them, bacteriophages can reduce the numbers of that specific bacteria, all while leaving others unharmed (17).

Table 1. Properties acquired through lysogenic conversion of bacteria mediated by temperate bacteriophages

Properties acquired through lysogenic conversion	Bacterial Host	Phage Conferring Advantage	Ref
Cell colonization and adhesion	*Vibrio cholerae*	VPIϕ	(18)
Cell invasion	*Salmonella enterica typhimurium*	Gifsy-1	(19)
	Streptococcus pyogenes GT8760	H4489A	(20)
	Staphylococcus aureus	Sϕ-C	(21)
Resistance to serum and phagocytes	*Escherichia coli*	Phage λ	(22)
	Pseudomonas aeruginosa 1	Phage D3	(23)
	Shigella flexneri	SfII	(24)
	Streptococcus pyogenes	SP24	(25)
Exotoxin production	*Clostridioides botulinum* Type C and D	c-st	(26)
	Escherichia coli (STEC)	933W	(27)
	Staphylococcus aureus	PS 42-D	(28)
	Streptococcus pyogenes CS112	CS112	(29)
	Vibrio cholerae	CTXϕ	(30)

4.6. Bacteriophages in Nature

As stated previously, bacteriophages are widespread in nature and are present in every environment where bacteria are present, in a never-ending interaction with the bacterial community.

Not surprisingly, the human body also constitutes a source of bacteriophages. Bacteriophage profiles vary significantly from individual to individual and as a function of the region in the body they inhabit (oral cavity, lungs, skin, gut, and genitourinary organs) (31). Only a small number of phages are considered “constants” in the human microbiota (10). Some of the main conditioners of bacteriophage profiles are genetics, age, diet, and health/disease status. It is well established that the most drastic changes to the human microbiota (and its bacteriophage fraction) take place during infancy, a key developmental period. As shown in Figure 3, there is a rapid growth and expansion of all bacteriophage families, followed by a stabilization of the population of *Podoviridae, Siphoviridae* and *Microviridae* phages (that is kept throughout life).

On a different course, *Myoviridae* phage steadily decrease as the human develops (10). Another know fact is that certain pathologies can give rise to altered microbiological patterns (10) and consequently to modifications in the bacteriophage profile. Crohn's Disease (CD) and Ulcerative Colitis (UC) are two diseases that give rise to significant changes in the bacteriophage composition. In both cases, the predominant bacteriophages are *Caudovirales* phages, which increase in relative abundance, comparatively to the regular bacteriophage population (32). Despite the high abundance, patients with CD and UC demonstrate a decreased diversity and evenness of *Caudovirales* phages in the gut mucosa (33).

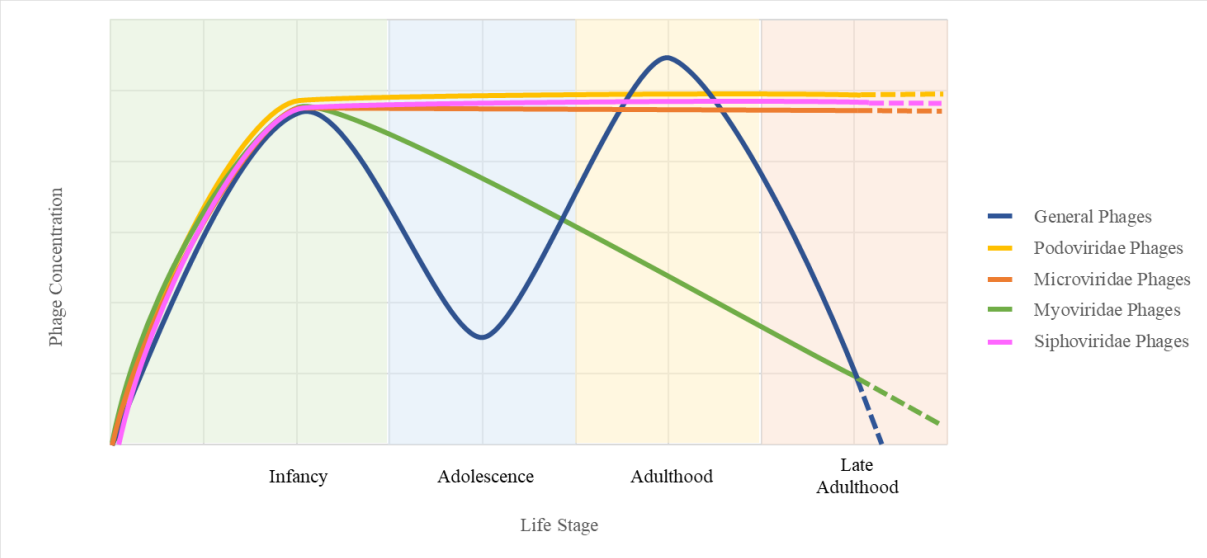

Figure 3. Changes in bacteriophage composition within the human gut throughout life. Graphic created with data reported in (10).

In general, in a healthy human gut environment, the most predominant bacteriophages belong to the order *Caudovirales* (families *Myoviridae*, *Siphoviridae*, and *Podoviridae*) and the families *Microviridae* and *Inoviridae* (34). The composition of a healthy adult gut phageome, as well as other eukaryotic viruses is present in Table 2.

Table 2. Composition of an adult-like gut virome

Bacterial Viruses	Eukaryotic DNA viruses	Eukaryotic RNA viruses
dsDNA phages:	Anelloviruses	Plant viruses
Caudovirales	Herpesviruses	Sapoviruses
ssDNA phages:	Adenoviruses	Rotaviruses
Microviridae		Coronaviruses

Note: Table adapted from (5)

5. Human Gut Microbiota

The Human microbiota has been continuously evolving with the Human species since its infancy. It can be defined as a group of commensal, symbiotic, and pathogenic bacteria, viruses (which include bacteriophages), fungi, and other microorganisms, that maintain relationships with one another to sustain their host's homeostasis (35). Any alteration to this homeostasis results in a phenomenon called dysbiosis which, left uncontrolled, can have severe consequences on the host's health and the overall microbiota (36). Within the microbiota, one can observe several elements from each of the three main domains of life: (i) Bacteria, (ii) Archaea; and (iii) Eukarya. Specimens of each of these domains are present in different proportions, the main ones being Bacteria and Archaea (37).

The whole Human microbiota is comprised of more than 30 million microorganisms (amount to 1-3% of the total human body weight). The habitats for these microorganisms can range from the skin to the gastrointestinal, respiratory and genitourinary tracts (38).

5.1. Structural Organisation and Function of the Human Gut

The gastrointestinal tract is one of the largest systems of organs in the human body. It includes the oral cavity, teeth and tongue, the pharynx, the salivary glands (lingual, submandibular, and parotid), the oesophagus, the stomach, the small intestine, the large intestine, the liver, the pancreas, and the gallbladder. The main function of the gastrointestinal tract, as well as the digestive system, is the turnover of food into nutrients and energy, for basic human survival. (39).

Bacterial and bacteriophage communities are most predominant in the gut due to a particular structure called the mucin layer. The main constituents of this structure are mucin glycoproteins, composed of tandem repeats of amino acids with exposed hydrophobic residues, interspersed with regions of high O-link glycosylation (hydrophilic sites). Alongside glycoproteins, in this mesh, one can also find other proteins, DNA, and cellular remnants. Depending on the organism and the body region in which the mucin layer is found, the thickness can vary from ten to 700 micrometres (40).

5.2. Composition of the Human Gut Microbiota

The human gut environment is considerably different from the marine one (where the highest concentration of bacteriophages can be found). This is in part due to several factors such as: major anatomical differences at the macro- and microcellular levels; action of the local immune system; constant inflow of new bacteriophages and bacterial hosts (from the external environment); and complexity of the human diet (4,37).

The microbiota is an everchanging structure. It doesn't remain equal throughout the entirety of human life. Factors like diet, age, genetics, and health/disease status help shape and regulate it (38). As a person develops and grows, so does the diversity of bacteria colonizing the gut Interestingly, as bacterial diversity increases, bacteriophage diversity decreases, only stabilizing in adulthood (Figure 4). When compared to adults, newborn babies have an almost sterile gut environment. During infancy there is a rapid expansion of the microbiota which tapers with adolescence and subsequently with adulthood (37). Colonization occurs right after birth. These birth conditions have been proved to impact the final composition of the microbiota. After comparing faecal samples of various babies with their respective mothers, Thursby et al. 2017 found that vaginally-delivered babies shared a great percentage of gut microbiota with their mothers (in 72% of cases) compared to Caesarean-section delivered babies (only 41% of samples where significantly similar between mother and child) (36).

On a similar remark, early breastfeeding also shows signs of regulating microbiota composition. Through the act of breastfeeding, this habitat is quickly colonized by various bacteria and bacteriophages (among other microorganisms). The first microbes to inhabit the gut of breastfed infants belong to the *Bifidobacterium* and *Bacteroides* species (37,38).

It is believed that by 2.5 years of age, a child's microbiota is, in terms of composition and function, comparable to an adult's one. Despite this, after reaching the age of 65, microbiota activity starts to decline. With this decline, it is possible to observe behaviours such as the decrease in the metabolization of short-chain fatty acids (SCFA) and the increase in proteolytic activity. SCFA are used in the Human body as metabolic and immunologic mediators. When combining these factors, one can understand the elevated incidence of inflammation processes in older individuals (a phenomenon called "inflame-ageing") (36). Age also seems to be corelated with changes in the lytic-lysogenic phage ratio, although further studies need to be conducted (5). This is due to increased stress levels (such as inflammation) which can be observed, for example, in cases of Inflammatory Bowel Disease (IBD) (41).

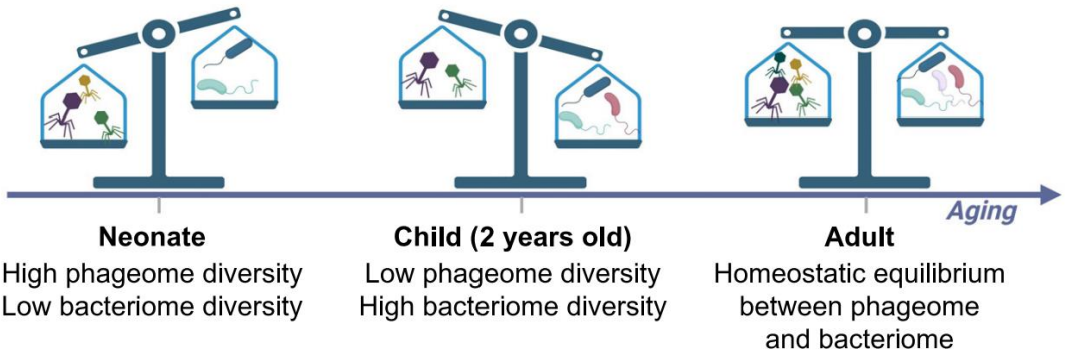

Figure 4. Changes in the ratio of bacteriophage to bacteria abundance as a function of age. Adapted from (5).

Concerning the role of diet in shaping the microbiota, one must keep present that living organisms only grow and thrive where sufficient nutrients are available. After comparing nutrient availability between the small and large intestines, it is easy to note a disparity in the quantity and quality of nutrients. In the small intestine, where nutrients are few and scarce, the only microorganisms capable of prospering are those that can metabolise simple sugars. In opposition, in the large intestine, an environment rich in nutrients such as fibres and microbiota-accessible carbohydrates, a completely different microbiota profile arises with a wider variety of microorganisms (36). Recently there has also been much debate about whether veganism (an all-plant-based diet) and other extreme plant-based diets can positively impact an individual's health. According to recent studies, neither all-plant-based diets nor all-meat-based diets have proven to be a health benefit. Some cases have even shown possible dysbiosis with severe alterations in the gut microbiota (36).

Other circumstances that can also lead to changes in microbiota composition and density are acidity levels, oxygen levels and the presence of antimicrobial substances along the gut, as seen in Figure 5. The small intestine shows a much lower pH (more acidic environment), greater oxygen levels, and higher concentrations of antimicrobial compounds when compared to the large intestine. All these factors act as inhibitors to microbial growth. In combination with high transit times in the small intestine, the end result is a habit that almost exclusively selects facultative anaerobes capable of adhering to the epithelial mucus layer. An example of one of the few groups of organisms selected by this environment are *Lactobacillaceae*. In opposition, in the large intestine, where the pH becomes more alkaline and the nutrients are abundant, there is more room for other microorganisms to grow and to take advantage of a larger array of energy

sources. Such microorganisms belong to the families *Prevotellaceae*, *Lachnospiraceae*, and *Rikenellaceae* (36).

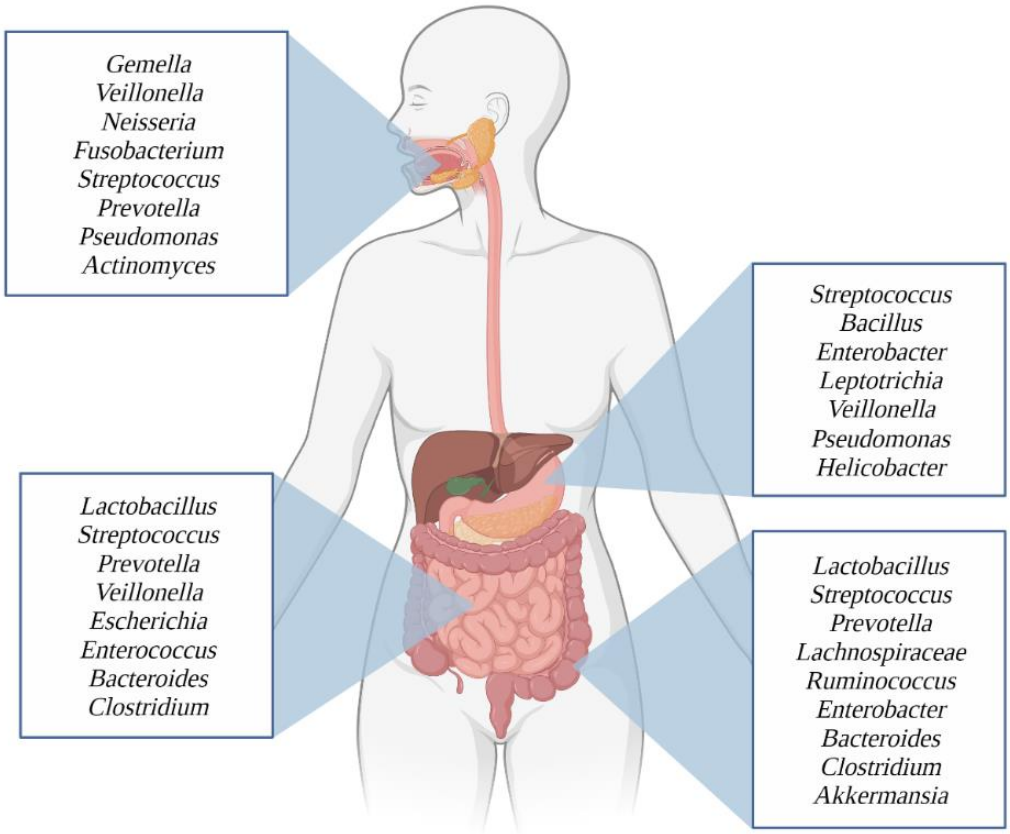

Figure 5. Human microbiota composition changes according to the location in the GI tract.
Adapted from (38).

After knowing that many factors can impact the microbiota, it's not difficult to see the richness and high complexity of this so-called "superorganism".

Although viruses are commonly overlooked when discussing the microbiota, these entities are also an important part of the gut microbiota, comprising both eukaryotic and prokaryotic viruses (Table 2). The virome is mainly composed by bacteriophages, mostly temperate phages. However, as we have come to see, this does not dismiss the existence of virulent (lytic) phages in the gut. When comparing the rate at which each of these types of phages evolves, it can be found that temperate phages have a considerably lower rate of evolution than virulent ones, once again proving the constant relationship with bacteria (lytic phages need to constantly evolve to be able to infect bacteria, despite resistance mechanisms) (37).

The predominant bacterial phyla in the human gut are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Of these six, the Firmicutes and Bacteroidetes phyla account for approximately 90% of the gut bacteria. Among these two

phyla, the most relevant bacterial genera are: *Lactobacillus*, *Bacillus*, *Clostridioides*, *Enterococcus*, *Ruminococcus*, *Bacteroides*, and *Prevotella* (only the last two belong to the Bacteroidetes phyla) (42).

5.3. Role of the Microbiome in Human Health

Some of the main functions of the microbiota include: (i) regulation of host metabolism; (ii) development of host's immunity; (iii) development of host's brain function (particularly during ontogenesis); (iv) protection against external pathogens (including opportunistic bacteria); and (v) homeostasis of local (gut) immunity (37). All these functions are carried out through a group of genes, encoded only in the microbiome (microbiota's genome). These genes code for specific proteins involved in the synthesis of nutrients and the metabolism of amino acids, carbohydrates and lipids (35). As stated before, one of the bacteriophage's role in nature is shaping the microbial composition, driving bacterial diversity and facilitating of horizontal gene transfer (2). Thus, changes in the microbial composition affect the normal equilibrium ou the human host, which can lead to disease states.

6. Bacteriophage-Microbiota Interactions

The many types of interactions between bacteriophages and bacteria can be divided into two main categories: (i) antagonistic; and (ii) mutualistic. An example of an antagonist interaction is the lytic cycle of phages, which results in the death of the host cell. On the other hand, one example on a mutualistic interaction is the lysogenic life cycle. As explained before, with this cycle bacteria can gain beneficial characteristics, becoming more suited to survive in their environment (13).

These forms of bacteriophage-bacteria interactions can be manifested as three types of dynamics: (i) Red Queen dynamics (RQD); (ii) kill-the-winner dynamics (KWD); and (iii) piggyback-the-winner dynamics (PWD). The RQD is mainly characterized by a parasitic relationship between the phage and the bacterial host that leads to the development of resistance (from the bacterium part). By following KWD, phages are more likely to predate on fast-growing and high-density populations, when compared to slow-growing and low-density ones. This kind of dynamic results in a long-term "levelling-out" of the overall bacterial population due to the "elimination" of the fast-growing strain. The KWD serves almost like a method of controlling bacterial population. In PWD, similarly to KWD, phages infect dense and fast-growing populations of bacteria. In PWD this infection is carried out by temperate phages who confer resistance to superinfection through lysogeny. This allows for the expansion of the lysogenic bacterial subpopulation (10).

The bacteria-bacteriophage balance is not the only result of the mechanism of phage infection, but also bacteria-resistance and phage counter-resistance. To guaranty their survival bacteria have developed several phage defence mechanisms. The current known host defence mechanisms are: (i) expression of different (or mutated) cell surface receptors; (ii) expression of S-layer proteins; (iii) production (or synthesis) of protective cell surface polysaccharides; (iv) production of outer membrane vesicles (their function is to entrap invading phages, lowering the chances of infection by reduction of viral load); (v) restriction/modification system; (vi) abortive infection mechanisms ; and (vii) the recently identified clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein (Cas) System (13,43–48). It’s important to note that none of these mechanisms is completely infallible. It has been reported that some bacteriophages have the ability to overcome the host’s CRISPR-Cas Systems by deletion or mutation of specific CRISPR-Cas acting sites or even by expressing anti-CRISPR proteins (2). If only bacteria had the ability to develop resistance mechanisms, no

bacteriophage could persist in an environment for long periods of time. Thus, bacteriophages developed their own counter-resistance mechanisms. These include: (i) production of glycosidases to degrade host capsule, exposing binding sites; (ii) expression of mutated or highly-variable binding proteins; (iii) mutation or deletion of CRISPR binding sites; and (iv) expression of anti-CRISPR proteins (2).

As in every ecosystem, any imbalance of these mechanisms can have significant consequences. Given the important role of the gut microbiota in the human health, even a small alteration in the population of either of these entities or changes in their biological mechanisms can lead to pathological states.

6.1. Bacteriophages and Gut Diseases

When discussing diseases, it's still hard to distinguish if alterations in the gut virome/microbiota are at their cause or if they are a consequence/effect of the same (34). Despite this, in recent years several studies have observed a correlation between alterations in the intestinal microbiota, including bacteriophage composition and certain types of diseases, such as: Inflammatory Bowel Disease; Ulcerative Colitis; Crohn's Disease; *Clostridioides difficile* infection (CDI) (49); obesity (50); type-1 and type-2 *diabetes mellitus* (T1DM and T2DM, respectively); liver diseases (51); colorectal cancer (CRC); malnutrition (52); several neurological disorders; and recently also observed in a SARS-Cov-2 infection (53,54). Most of the diseases mentioned previously are related to changes in abundance and diversity of *Caudovirales* phages (34). However, depending on the type of disease, it is expected to find different ratios for these types of viruses as well as for commensal bacterial (2,55).

As mentioned before, bacteriophages are also implied in modulating immunological responses in the human organism. Some authors theorize that bacteriophages possess specific binding sites for proinflammatory mediators. According to Miernikiewicz et al. 2016, tail adhesin gp12 (expressed by T4 phages) can bind to *E. coli* cells through the surface lipopolysaccharide (LPS), preventing the production of LPS-induced proinflammatory cytokines (56). This phenomenon is due to the fact that LPS derived immune activation is dependent on its structure. By binding to the core region of LPS, T4 phages change LPS configuration enough to hinder immunostimulation (56). In another study conducted by Miedzybrodzki et al. 2008, T4 phages also showed the ability to reduce the production of

reactive oxygen species (ROS) by peripheral blood polymorphonuclear leukocytes, again by hindering LPS stimulation (57).

Besides having down-regulating effects on proinflammatory cytokines, bacteriophages can also up-regulate certain anti-inflammatory cytokines. In a study performed by Van Belleghem et al. 2007 *S. aureus* ISP and *P. aeruginosa* PNM, LUZ19, 14-1, and GE-vB_Pae-Kakheti25 phages proved to have predominantly anti-inflammatory effects. This was due to the up regulation of the interleukin 1 receptor antagonist (*IL1RN*) gene and reduction of C-X-C Chemotactic Ligand 1 and 5 (*CXCL1* and *CXCL5*) (58).

In a more gut oriented view, Barr et al., 2013 showed that phages may be implied in a new sort of immunity, a bacteriophage-mediated immunity, also called Bacterial Adherence to Mucus (BAM) immunity (40). One example are T4 like phages which possess Ig-like Hoc proteins in their capsid (5). In this kind of immunity, these phages, linked to the mucin layer of the gut by immunoglobulin-like protein, predate on external bacterial pathogens, killing them before they can even colonize the gastrointestinal lumen, almost like a "first line of defence", as represented in Figure 6 (5,35,37,40). In Figure 6 it is also represented the passage of bacteriophages into the circulation, directly stimulating and influencing the immune system. In the bloodstream, phages are recognised by innate immune cells, which leads to the release of cytokines (such as interferon-γ). In the mucus layer, these entities are found in the form of free phages, as opposed to prophages (59).

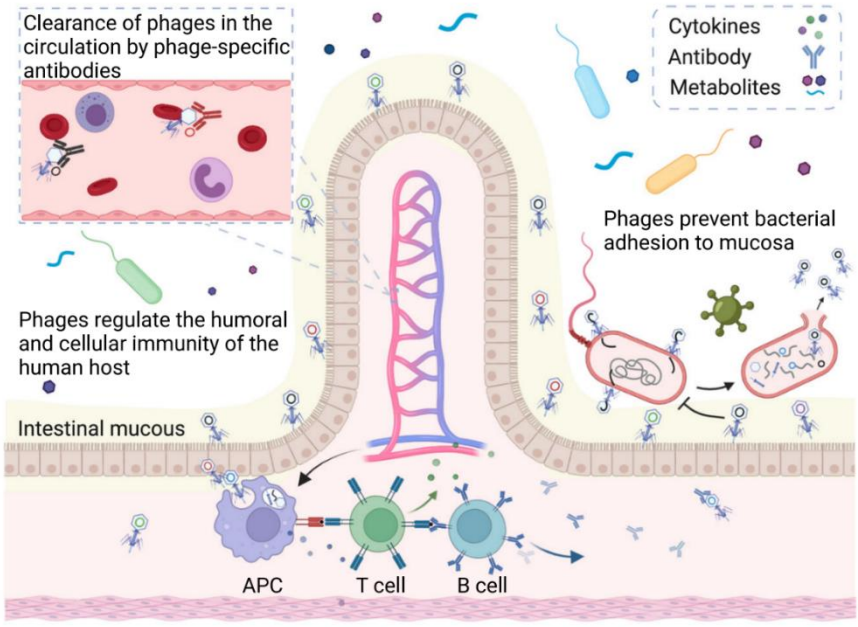

Figure 6. Interaction between bacteriophages and the human gut immune system, as well as existing gut bacteria. Adapted from (5).

6.1.1. Type-1 and Type-2 *diabetes mellitus*

Diabetes mellitus is a group of metabolic pathologies with multiple possible aetiologies characterized by a chronic elevation of glycaemic levels. In this group of illnesses, it is common to observe changes in carbohydrate, lipid, and protein metabolism. According to the American Diabetes Association, there are three types of *diabetes mellitus*: (i) type-1; (ii) type-2 ; and (iii) gestational diabetes. Type-1 *diabetes mellitus* is characterized by the destruction of insulin-producing beta-cells (located in the pancreas), resulting in the total absence of insulin. These individuals rely on exogenous insulin to survive. The main symptoms of T1DM include polyuria, polydipsia, polyphagia, and rapid weight loss (60). T1DM can also be defined as an immune-mediated chronic inflammation process. As a result, many researchers consider T1DM to be an autoimmune disease (61). Despite its pronounced genetic/hereditary component, external factors such as diet can heavily shift the prognosis and disease progression of T1DM. Proof already exists that high-fat, carbohydrate-rich diets, such as the typical Western diet, accelerate disease progression (62). This worsened prognosis is evidenced by a decrease in *Prevotella*, *Faecalibacterium*, *Eubacterium*, *Fusobacterium*, *Roseburia*, *Anaerostipes*, and *Subdoligranulum* populations, and an increase in *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Streptococcus* specimens (35).

Besides bacteria, viruses and bacteriophages can also be implicated in T1DM. The *Circoviridae* family appears to be the most common viral family associated with T1DM. In what regards to bacteriophages, *Myoviridae* phages are shown to decrease in abundance in T1DM, and an increase in amyloid-producing *E.coli* and respective phages seems to be a predisposing factor (35). In a study conducted by Tetz et al. 2019, in individuals with T1DM, 63 types of *E. coli* bacteriophages (both temperate and strictly lytic) were identified by the authors (63). The same study also reported a positive correlation between the increase of temperate *E. coli* phage particles and the decrease of their respective hosts. The authors suggested that this increase might result from a prophage excision, although it was not clear what factor triggered induction. The authors also concluded that these bacteriophages belonged to the *Peduovirinae* subfamily in the *Myoviridae* family and unclassified *Lambdavirus*, in the *Siphoviridae* family. According to this study, these bacteriophages stimulate the production of curli amyloid fibre, which forms highly immunogenic complexes with DNA. These complexes can later stimulate toll-like receptors 2 and 9 (TLR2 and TLR9), inducing immune reactions, by producing type-1 interferon. This immunogenic marker can then trigger pancreatic beta-cell

death (by deposition of said complexes in the pancreas), ultimately leading to the development of T1DM (63).

Type-2 *diabetes mellitus* is the most common type of diabetes. It usually manifests itself after 40 years of age, and individuals generally do not require exogenous insulin. This type of diabetes is strongly associated with obesity, smoking, high blood pressure, and dyslipidaemia, being more prevalent in developed countries like the United States of America. T2DM is the result of continuous resistance to insulin. Altered microbiomes have also shown to be, in some way, correlated to the development of T2DM (60). The most common observable changes are decreases in the populations of *Firmicutes* and *Clostridia* order and increases in *Escherichia, Clostridioides, Lactobacillus, Pseudomonas,* and *Staphylococcus* phages (35). In the gut, Ma et al. 2018 found once more, predominantly *Caudovirales* phages (*Siphoviridae* – 37%; *Myoviridae* – 21.4%, *Podoviridae* – 6.7%, and unclassified *Caudovirales* families – 34.9%) (64).

As stated previously, one of the major diseases related to T2DM is obesity. When studying the relationship between bacteriophages and this pathology Schulfer et al. 2020 found consistent decreases in the population of *Siphoviridae* phages and increases in *Microviridae* phages and *Phycodnaviridae* and *Mimiviridae* viruses (eukaryotic viruses) (65).

6.1.2. Inflammatory Bowel Disease

Inflammatory Bowel Disease is an immune-mediated disease characterized by chronic inflammation of the gastrointestinal tract. IBD is an umbrella term that encompasses illnesses such as Crohn's Disease and Ulcerative Colitis. Many factors are implicated in the development of IBD, with the most important being genetics, the immune system, diet, and gut microbiota (35). In every case of IBD, it's common to note a population decline of *Faecalibacterium prausnitzii* and species of *Roseburia, Bifidobacterium,* and *Lactobacillus*. All these bacteria are considered beneficial and essential to the correct functioning of the gut. With this unbalance, there is an opposing increase in pathogenic bacteria such as *E. coli*, *Clostridioides difficile*, *Oscillospira*, and unclassified *Ruminococcaceae* (35).

Specific profiles of *Caudovirales* phages are believed to be the base for the development of most cases of UC and CD and IBD, in general. According to Zuo et al. 2019, in most cases of UC, one can observe a higher title of *Escherichia* and other *Enterobacteria* phages, although the reasoning for this is still to be uncovered (66). On a similar remark, Clooney et al. 2019 found that in cases of general IBD, the relative abundance of *Caudovirales* was indeed

increased and that there was an increased relative abundance of temperate bacteriophages, having their lytic life cycles triggered by excessive amounts of inflammatory activity (67).

6.1.3. *Clostridioides difficile* Infection

Infection by *Clostridioides* (formerly *Clostridium*) *difficile* is slowly but surely becoming one of the most concerning nosocomial infections, affecting mainly older adults and overall bacterial infections to this day. In the United States of America more than 500,000 new cases and 29,000 deaths are reported every year (68). One of the main risk factors for *C. difficile* infection is the misguided use of antibiotics. As a consequence of their, the endogenous gut microbiota is disrupted, causing it to lose the ability to naturally repress *C. difficile* specimens (68).

In cases of CDI, it was observed a high relative abundance of *Caudovirales* phages and *Anelloviridae* viruses (eukaryotic viruses), along with low relative abundances of *Microviridae* phages (5).

6.1.4. Colorectal Cancer

One of the main problems that emerge in patients suffering from Colorectal Cancer is the out of proportion growth of pathogenic organisms. These organisms can cause grave alterations to the fragile balance of the gut microbiota, worsening the prognosis of the disease. Two examples of such pathogenic microorganisms are *Fusobacterium nucleatum* and Adherent Invasive *Escherichia coli* (AIEC). A particular strain of AIEC, *E. coli* NC101, has demonstrated the capability of enhancing tumour growth in animal models prone to inflammation (59). In a study performed by Gogokhia et al. 2019, to prove the efficacy of phage therapy in treating CRC patients, positive for *E. coli* NC101, APC^{min} mice were colonized with said bacteria and then subsequently inoculated with three types of anti-*E. coli* NC101 phages: (i) Phage NC-A; (ii) Phage NC-B; and (iii) Phage NC-G. Continuous administration of this "phage-cocktail" proved to reduce *E. coli* colonization, which resulted in a reduction in tumour sizes and in an improvement in survival rates. Subsequently, a transcriptomic analysis showed a decrease in tumour markers such as metastasis-associated and invasion-associated genes (59).

In this same study, Gogokhia also proved that in phage treated animals, the reduction of tumour markers is accompanied by an increase in the expression of immune system transcripts, which manifests itself through an increase in $CD4^+$ and $CD8^+$ T-cells count. The rise in T-cell

counts can occur via one of two mechanisms: (i) *E. coli* lysis releases immunogenic bacterial components that activate the immune system; and (ii) bacteriophages directly stimulate the immune system. There is still an information gap, especially when it comes to *in vivo* studies, relative to this second mechanism. The so-called "direct" mechanism through which phages activate the immune system is, in actuality, an indirect stimulation of $CD4^+$ T-cells. Generally speaking, in mammalian immune systems, T-cell activation requires interaction with dendritic cells (antigen-presenting cells). Dendritic cells, which possess Toll-like receptors, can internalize phages via endocytosis (these viruses are incapable of infecting eukaryotic cells by themselves) and subsequently dismantle their capsids, exposing the genetic material (59).

6.1.5. Other Diseases

Besides bacteria, some authors also note the possibility of bacteriophages directly interacting with eukaryotic cells and proteins (34). Some authors believe that prion-like domains of certain bacteriophages when in contact with eukaryotic proteins, can lead to protein misfolding and ultimately cause diseases such as Alzheimer's, Parkinson's, and Amyotrophic lateral sclerosis (autoimmune and neurodegenerative diseases). These prion-like domains were found in many *Caudovirales* phages, especially in *Myoviridae* phages (69).

6.2. Gut-Microbiota-Brain Axis

The gut-microbiota-brain axis is a term used to define the bidirectional communication pathway between the human gastrointestinal tract and the central nervous system, especially the brain (70). This communication system is comprised of two components: (i) the autonomic nervous system (ANS) and vagus nerve (VN) (which provide a direct form of communication); and (ii) the enteric nervous system (which makes up the bridge between the gut and the ANS/VN, within the spinal cord). Any kind of damage or abnormality in any of the components shows serious repercussions at the intestinal level (such as impaired intestinal reflexes and external neural control) (70). Besides the gastrointestinal and nervous systems, this axis also implicates the endocrine, immunologic and metabolic systems (70).

The main impact of the gut in shaping the neuroendocrine system is observed by the stress response. Early exposure to stressing factors (such as mother-separation in mice) leads to the exaggerated production of interleukin-6 and monocyte chemotactic protein 1, which are directly

implicated in the colonisation and expansion of *Enterococcus faecalis* and *Pseudobutyrivibrio* in the gut (70). Other observable changes include the decrease of the population of *Bacteroides* and the increase of *Clostridioides* species (70).

The development of the immune system in the gut is deeply rooted in the interaction between microorganisms and TLRs. These TLRs are ubiquitous in the immune system but they can also be present in neurons. This way, the nervous system can directly interact with gut microorganisms (70).

Lastly, changes in commensal gut microbiota can impair the synthesis of essential nutrients derived from bacterial metabolism. These bacterial by-products include short-chain fatty acids, vitamins, and compounds that can act as neurotransmitters, namely serotonin and gamma-aminobutyric acid (GABA). These neurotransmitters communicate with the central nervous system via the vagus nerve, which serves as the primary link between the gut and the brain. Thus, disruptions in serotonin and GABA production result in impaired neurological pathways and, as a direct consequence, various nervous system disorders (70).

An example of such a disorder is autism spectrum disorder (ASD). ASD is a heterogenous, multi-factorial, neurodevelopmental disease. It's characterized by impaired social skills, such as communication and social interactions, as well as increased repetitive behaviours (70). Other conditions commonly co-occurring with ASD include epilepsy, Down Syndrome, and Fragile X syndrome (70). ASD has a strong genetic component, but despite this fact, no mutation has been pointed out as a "universal trigger" for the disease. However, all occurring mutations in ASD seem to be somehow related to disrupted synaptic pathways (70).

Refusing to eat certain types of food is a common occurrence in children with ASD, given their behavioural impairments. As a result, their food repertoire is severely limited, with little nutrient variety (70). Given the previously mentioned role of the diet in shaping the microbiota, it comes as no surprise the high incidence of gastrointestinal problems in children with ASD. These gastrointestinal disorders appear to be due to a compromised intestinal barrier, also known as "leaky gut". With this condition, dietary products and even bacterial metabolites can pass through the gut epithelial barrier, enter the bloodstream, and possibly reach the nervous system and/or brain (70).

To further test the role of bacteriophages and diet in neurological processes and diseases, Mayneris-Perxachs et al., 2022 studied various types of diets and gut bacteriophage profiles, trying to establish a connection with cognitive processes such as memory and executive functioning (71). At first glance, Mayneris-Perxachs et al., 2022 found that in comparison to low-fat diets, high-fat diets gave rise to a bacteriophage profile much richer in lytic

Microviridae phages, with decreases in *Siphoviridae* phages (temperate phages) (71). In another aspect of the study, Mayneris-Perxachs et al., 2022 discovered that individuals with high levels of *Caudovirales* phages and, in particular, high levels of *Siphoviridae* phages had better results in cognitive tests. *Siphoviridae* phages were associated with significantly better short- and long-term memory tests, while general *Caudovirales* phages were associated with better scores in executive functioning tests. Oppositely, *Microviridae* phages were associated with worse results in both types of tests (71).

7. Bacteriophages as New Therapeutic Approaches

Phage therapy is defined as the direct administration of virulent bacteriophages to a patient, in order to lyse a specific bacterial pathogen that is causing a clinically relevant infection (72). This therapy is not a completely new approach to treating specific diseases, having been used for many years as "bacteriophage cocktails", to treat illnesses such as cholera and dysentery. However, in both these cases and many others, antibiotics still impose a more efficient (except in multi-resistant strains of bacteria), reliably, and cost-effective treatment option (13,59). In order to try and reduced these variables and improve treatment success, some aspects of phage therapy like the exclusive use of strictly lytic phage and confirmed antimicrobial activity towards the target pathogen have already been standardized (72). In Table 3 are summarized some the advantages and disadvantages of phage therapy, in comparison to mainstream antibiotic therapy.

Table 3. Advantages and disadvantages of phage therapy in comparison to mainstream antibiotic therapy

Advantages	Disadvantages
Does not kill commensal microbiota	Need for bacterial identification, due to the narrow activity spectrum
After bacterial host's death, phage action ceases	Induction of phage-neutralizing antibody production
Tolerated by patients with antibiotic allergies	Small body of evidence from clinical trials
No effects over mammalian cells	Lack of regulatory and legal framework
In case of resistance development, phages can evolve alongside bacteria (Red-Queen dynamics)	
Antibiofilm activity	
Simple and inexpensive production	

Note: Table adapted from (73)

In modern medicine, bacteriophages take their place as possible prophylactics, diagnostic factors (disease biomarkers) and therapeutic strategies. Some of the many applications of bacteriophages include: detection of disease associated biomarkers (73,74); construction of matrices with potential applications in bioengineering and regenerative medicine (75); usage as components of biosensors able to detect abnormal human cells or microbial contaminants (76); design of vaccines against pathogenic agents such as nematodes (77) and protozoa (78); design

of new vaccine delivery systems for neurodegenerative disorders (79); creation of targeted drug carriers for emerging antibiotic-resistant infections (80); formulation of malignant cell-specific targeted gene/drug delivery systems for cancer therapy (81); and applications for treatment of multi-resistant bacterial infections, for which several succeeding examples have been reported (82,83).

Referring to the therapeutic applications of bacteriophages, several studies support its efficacy and relevancy. PDX is a strictly virulent bacteriophage belonging to the *Myoviridae* family that can infect Enteroaggregative *E. coli* (EAEC) cells. In a study by Cepko et al. 2020, the same bacteriophage seemed to reduce, in a dose-dependent manner, the level of EAEC in mouse faeces. PDX also demonstrated lytic activity towards EAEC when cultured anaerobically in the presence of human faecal bacteria (84). With this study, the authors suggest using such bacteriophages to treat EAEC infections in children in developing countries (a region deeply troubled by EAEC-mediated traveller's diarrhoea) (84).

The efficacy of bacteriophages therapy in treating pathogenic AIEC (strain LF82) was also demonstrated by Galtier et al. 2017. The authors showed that by administrating a bacteriophage cocktail comprised of LF82_P2, LF82_P6, and LF82_P8 phages, this bacterium, predominant in the ileal mucosa of individuals affected by CD, could be selectively killed, improving the prognosis of the disease (85).

According to a study performed by Duan et al. 2019, individuals affected by alcoholic hepatitis have more *Enterococcus faecalis* specimens in the gut microbiota (2,700 fold more compared to healthy controls) (86). Infection by *E. faecalis* seems to be linked to the induction of mild hepatic steatosis and exacerbated ethanol-induced liver disease (by mechanisms still to be uncovered) (86). This bacterium produces cytolysin, an exotoxin that has lytic activity against not only Gram-positive bacteria but also eukaryotic cells. In this study, the number of cytolysin-producing *E. faecalis* present in mice gut with alcoholic hepatitis, was reduced after infection with a virulent *Podoviridae* phage, resulting in the improvement of the outcome of the disease (86).

Another example of a novel therapeutic approach using bacteriophages as a tool for combating rising diseases is modifying temperate bacteriophages into virulent phages, killing bacteria more effectively. As mentioned, selected bacteriophages for phage therapy need to be strictly lytic. To overcome the shortage of virulent phages for some bacterial species, engineering approaches are being used to modify temperate phages into virulent ones. In a study by Selle et al. 2020 temperate bacteriophages for *C. difficile* were genetically modified to become strictly lytic phages, carrying a modified CRISPR-Cas3 system (directed at the genome

of *C. difficile*) (68). In this study, the authors proved that this new approach was indeed efficient in reducing the gut colonization of *C. difficile* in mice. This reduction of bacterial numbers can be due to two mechanisms of the now lytic phages: (i) irreversible DNA damage by expressed type I-B Cas effector proteins (directed by CRISPR RNA); and (ii) holin and endolysin expressed during phage lytic replication (68).

Another important aspect in the use of bacteriophages for therapeutic applications is the specificity in the delivery to the site of infection. In a study conducted by Zheng et al. 2019, the authors proved the efficacy of phage therapy in colorectal cancer, using encapsulated irinotecan (the first-line drug against CRC) within dextran nanoparticles covalently bonded to bacteriophages (A-phages) (87). These A-phages accumulated in CRC tumours of mice, eliminating *F. nucleatum* specimens and reducing tumour progression. *Fusobacterium* is implied in the formation of a pro-tumoral microenvironment that is highly chemoresistant and immunosuppressive. In clinical studies, abnormal proliferation of *Fusobacterium* induced the failure of chemotherapy. In an opposing way, SCFAs (produced by fermentative bacteria) have been shown to suppress the progression of CRC and induce anticancer immune responses. This therapy also proved to reduce therapeutical side effects due to targeting of normal healthy cells (very common in chemotherapy) (87).

Although phage therapy only applies virulent phages to kill bacteria, as natural killers, on account of kill-the-winner dynamics, only a certain number of pathogenic bacteria can be eliminated. Otherwise, there would be a halt in bacteriophage production due to lack of hosts. This way, humans still rely on a competent/functional immune system to overcome the infection. This relationship between phages and the immune system is termed “immunophage synergy” (72).

Despite all the advantages these new phage therapies bring, current microbiome therapies such as bacterial probiotics and antibiotics are not 100% advantageous. By introducing new opportunistic pathogens to the gut or by inhibiting the “natural” gut microbiome, these therapeutic strategies can further aggravate gut dysbiosis, proving to do more harm than good (5). The same can be said for bacteriophages. Although phages such as ankyphages (marine sponge’s bacteriophages) can “protect” bacterial cells from eukaryotic-type immune systems, in other cases, as stated before, through lysogeny or predation, bacteriophages can modulate and deplete GUT microbiota, affecting general homeostasis (13,88).

8. Conclusions

In recent years, a considerable amount knowledge regarding bacteriophages has been accumulated, due to a necessity in finding new therapeutic alternatives for combating multidrug-resistant strains of bacteria. New bacteriophage families were discovered, new classification systems were proposed, and new functions and roles in the human body and health were uncovered.

The rising number of studies in this field and novel research tools, including genome sequencing and metagenomic analysis have brought forth new information about the relationship between the gut microbiota and human diseases, especially the role of bacteriophages in the health/disease balance of the human body.

Future research is still needed and should include the development of new methods for characterizing bacteriophages in the gut (using new molecular genetic technologies), a better characterization of the parallel evolution of bacteriophages and bacteria in the gut (as bacteria evolve, so do phages and vice-versa), increasing the amount of evidence supporting and strengthening the role of bacteriophages in human diseases, and the development and standardization of new forms of therapy for fighting bacterial infections, utilizing bacteriophages.

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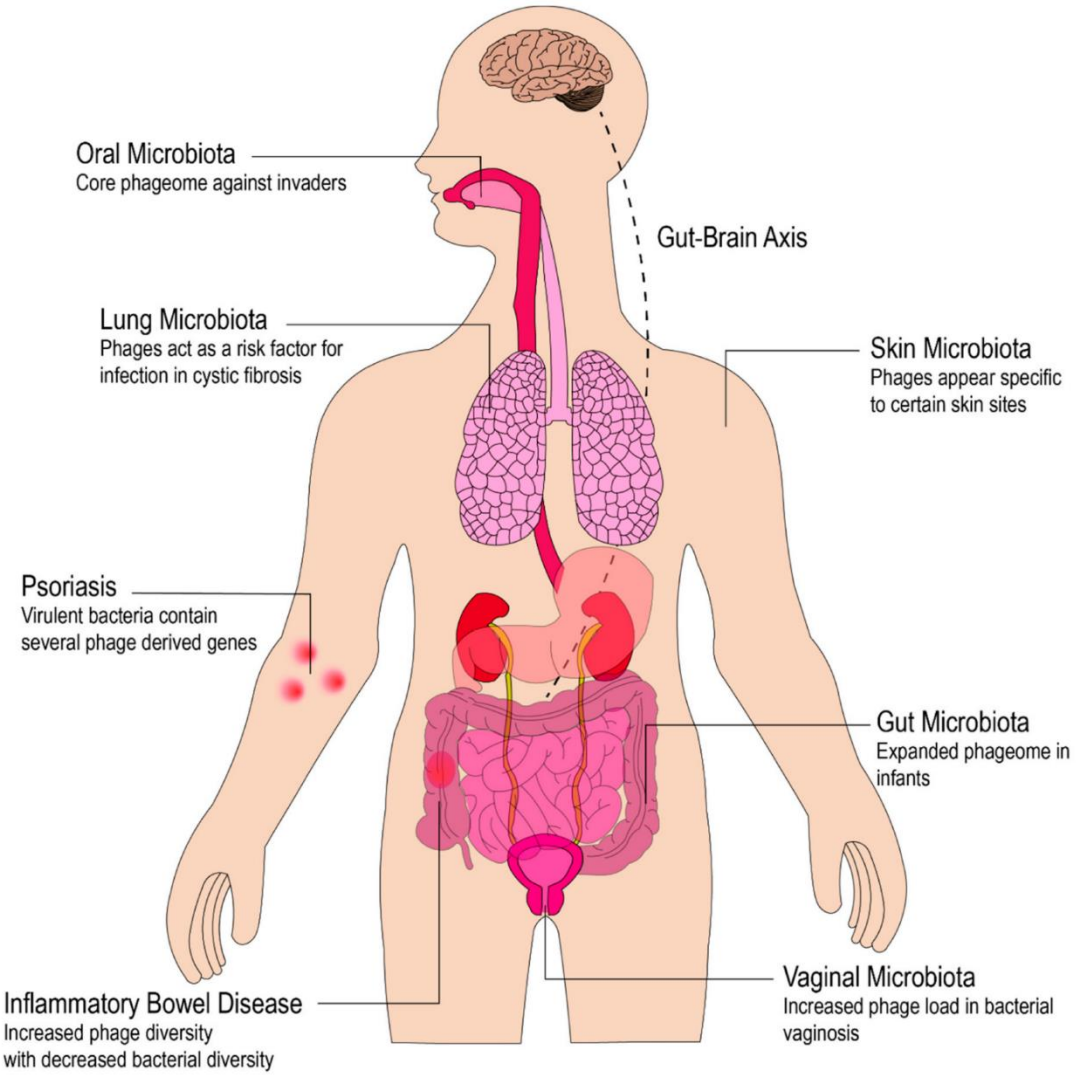
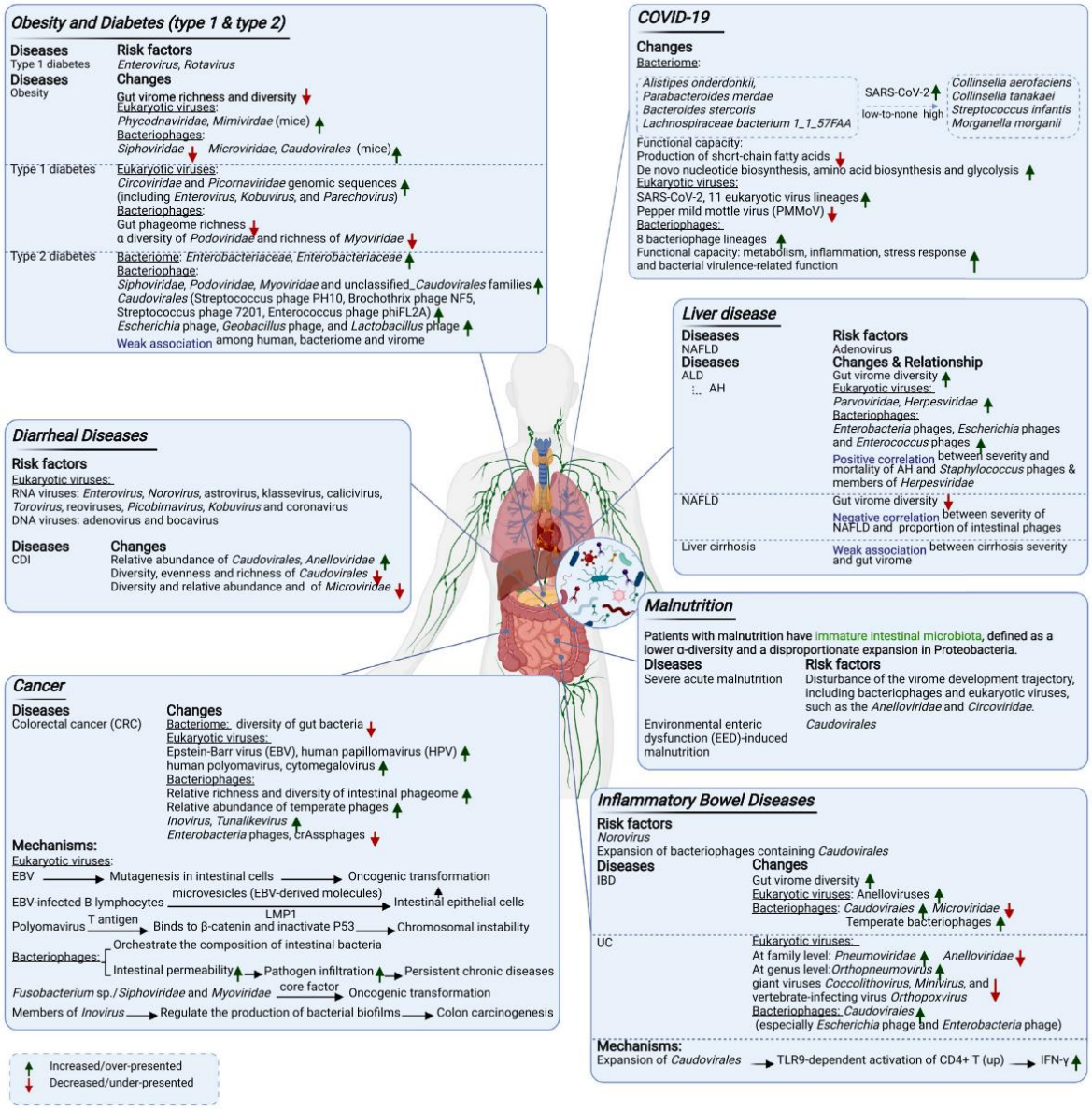
Annexes

A1. Diagram highlighting regions in the body in which phages have been indicated to play a role



Adapted from (34)

A2. The gut virome in human diseases



Adapted from (5).

A3. Studies of phages in the human body in different diseases

Disease	The Most Important Finding
Inflammatory bowel disease (IBD)	Expansion of *Caudovirales* phages in enteric virome of IBD patients
Ulcerative colitis (UC)	The detection of the abundance of *Caudovirales* phages in gut mucosa UC patients, whereas a decrease of evenness, diversity and richness of *Caudovirales* phages and an indication of dysbiosis in mucosal virome in UC patients
Gulf war illness (GWI)	GWI mice had decreased abundance of the *Microviridae* phage with increased abundance of the *Siphoviridae* and *Myoviridae* phage in the enteric viral population
Type 1 diabetes (T1D)	Predominance of temperate phages in the gut of children. The importance of diabetogenic *E. coli* prophages in the autoimmunity and T1D progression
Type 2 diabetes (T2D)	Increase in the number of gut phages in T2D adult individuals. Phages specific to *Enterobacteria*, *Escherichia*, *Lactobacillus*, *Pseudomonas* and *Staphylococcus*, were detected in T2D patients. T2D-related factors in the gut of T2D patients cause temperate phages to switch to the lytic cycle
Autoimmune and neurodegenerative disorders	Phages circulate in human biological fluids in neurodegenerative diseases. *Staphylococcus* phage and *Shigella* phage were detected in cerebrospinal fluid in neurodegenerative disorders. Prion domains present in phages may be involved in interactions with eukaryote proteins and in protein misfolding in humans and their connection with autoimmune and neurodegenerative disorders
Central nervous system infection	There were no trends in diversity in the viromes between cerebrospinal fluid specimens from individuals without and with central nervous system infections. The majority of phages of the cerebrospinal fluid were *Caudovirales*. Temperate *Myovirus* and *Siphovirus* phages were present in individuals without and with infection
Malnutrition	Reducing the diversity of gut viromes in children
Cystic fibrosis (CF)	The presence of filamentous *Pseudomonas* Pf phages in sputum may be associated with chronic infection and increased antibiotic resistance in CF patients

Adapted from (35)