A Critical Appraisal of Current Microbiological Hot Topics

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Edited by

T. G. Villa and Ana G. Abril

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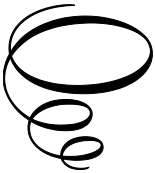

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FOREWORD

TOMÁS G. VILLA[1] AND ANA G. ABRIL[1,2]

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This is a multi-author book that, although at first glance may appear to encompass unrelated topics, upon close examination will immediately manifest the mental thread underlining its configuration. A common junction is the microbial world, as all the authors included in this publication share their interest in microbiology, although specializing in different aspects of this scientific study, as well as looking at it from diverse perspectives.

Accordingly, some of us are interested in the microbial life in a particular geological era, such as the Anthropocene, and the persistence of some of those microorganisms that currently remain in our planet. The maintenance of the Earth´s microbiome is crucial for all living beings but, although evolution is always present and creating change, the major changes to our planet are due to human activities that, albeit affecting our environment for thousands of years, have recently escalated and affect every living organism. This led Berg and Cernava (2022) to hypothesize that, in terms of diversity, a shift in the plant world has already occurred, and one of its characteristics is the decrease of host specificity. Theis and colleagues, in 2016, described plants as holobionts (a community including the host and its symbiotic microorganisms), in which the plant-associated microbial diversity is strongly connected with the biodiversity of plants, hence, the survival of one the members of the community is closely linked to the other's existence (Wasserman et al., 2019). According to Berg and Cernava, the botanical world is suffering from extinction rates of plant species that are currently 500 times higher than expected from 'natural' processes; hence, confirming that it is the result of human action. This anthropocentric behavior, results in the destruction of the natural world, either for the production of agricultural crops, to either

feed the ever-increasing human populations, or our domestic animals (Humphreys et al., 2019), or due to the pollution entailed by human activities. These are all subjects included here, in particular in the last chapter of this book.

Humans have always tried to tame microorganisms, even before they knew that such organisms existed, not only to take advantage of their metabolism in foodstuffs elaboration, by mainly to prevent pathogenic microbes from causing illness and death. Microbiology has played a major role in illness prevention, and cure, with a myriad of discoveries, ranging from the advancement of attenuated viral and bacterial strains, to the development of vaccines, a crucial breakthrough that, together with antibiotics, has helped, not only the quality of human life, but considerably extended human life-expectancy. All these aspects mentioned are included in the articles in this book, without forgetting the negative aspects, such as the discovery of antibiotic-resistant bacteria, suggesting possible ways to combat this current human health threat.

A usually neglected faced in microbial pathology is the subject of pathogenic fungi, as well as those organisms developing strains that are resistant to antifungal compounds, as it the case for bacteria; unfortunately there are still many unknowns in this area, resulting in failures in the treatment of fungal diseases. Fungal infections are not necessarily minor, as they result in thousands of deaths every year; therefore, these studies and related topics are also included in this volume.

The importance of the gut intestinal microbiota, that can produce serious medical conditions when in dysbiosis, is also recorded here, as these microorganisms are responsible for a variety of diseases that currently constitute a major health problem; these include the development of food allergies, as well as the role played by the gastrointestinal bacteria in the development of a variety of syndromes, including obesity.

Finally, due to space limitations, we had to limit the subject concerning the influence of microorganisms on industrial processes, to just the role played by bacteria in the oil industry, as currently world oil markets are in turmoil, not only from the effects of Covid 19 pandemia, but also due to the crisis created by the war in Europe.

Santiago de Compostela and Vigo (Spain) September 2022

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CHAPTER 1

BACTERIAL ATTENUATION AND VACCINES

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Abstract

Virulence attenuation, the 'weakening' of the pathogenic ability of an organism that prevents it from causing disease while still remaining alive, constitutes one of the most effective approaches in vaccine development against either bacterial or viral diseases. This technique has the advantage of eliciting good immunological memory responses, as compared to vaccines elaborated with dead bacteria, but also has the drawback of potentially being more dangerous, as some organisms can revert in virulence and cause disease. The phenomenon of virulence attenuation was discovered in the 19th century and still remains currently in use; present studies aim to develop new ways of attenuation, with the goal of generating less virulent bacterial strains that closely resemble the original pathogenic organism, hence capable of eliciting good immunity, but innocuous to the people who receive them as vaccines. The present chapter

reviews the use of relevant attenuated bacterial vaccines for the prevention of a variety of diseases, caused by either Gram-positive or Gram-negative bacteria.

Introduction

The term ‘attenuation’, in Microbiology, describes the techniques used to reduce/eliminate the pathogenic ability of a microorganism; this can either occur “naturally” (involving yet to discover extrinsic or intrinsic factor/s) or require treatment in the laboratory.

What is known as the “vaccination era” (the widespread use of vaccines around the world) started more than two centuries ago, although there is evidence that some cultures had been using vaccines for over 1,000 years. This widespread use of vaccines has been so successful that certain illnesses have been eradicated, and others controlled; these include infectious diseases that, in the past, have caused millions of human casualties. Smallpox is one on these success stories, this disease is considered as eradicated from Earth, despite the fact that the primordial reservoir of this poxvirus remains unknown. Smallpox probably represents the first disease documented to undergo virulence attenuation, due to the contributions of Edward Jenner to medical science in the 1790s (Plotkin, 2014). Of particular importance is the 14th of May of 1796, when Jenner successfully vaccinated an eight-year-old boy against smallpox; the boy was inoculated with the liquid obtained from cowpox blisters on his mother’s hands.

Jenner is officially recognized as the first person to successfully prepare, and use, an attenuated vaccine to protect against smallpox; in truth, there were a number of earlier reports that demonstrated the use of cowpox as prevention for the development of the smallpox disease; perhaps those accounts were dismissed, or ignored, because they lacked the scientific precision of Jenner’s publication (An Enquiry into the Causes and Effects of the Variolae Vaccinae, 1978). Among these vaccine researchers, that are not currently recognized, are the English ‘variolators’, people who exposed others to smallpox in order to promote immunity; ‘variolator’ is a word specifically coined for smallpox (variola virus) before the term vaccination was established. Prominent variolators include people like Thomas Dimsdale and John Haygarth; the latter was a physician who published an ambitious plan to exterminate smallpox in England. In addition, John Williamson (nicknamed John Notions) was a self-taught physician, that successfully variolated (protected against smallpox) at least

3,000 people in his native Shetland, Scotland, (Conacher, 2001). It is also worthwhile mentioning Benjamin Jetsy, a farmer from Yetminster (Dorset, England), who in 1774 used cowpox to successfully vaccinated his wife and two children against smallpox (Plett, 2006); as a result, some people consider Jetsy 'the first vaccinator'. Hammarsten and colleagues published an article in 1979 with the title: "Who discovered smallpox vaccination? Edward Jenner, or Benjamin Jesty?" Jacques Antoine Rabaut-Pommier, a French protestant pastor, was another of these pioneers in smallpox vaccination, around 1780 he established a link between smallpox, sheep pox and cowpox, and suggested the use of the animal poxes for inoculation, as they are less dangerous, to protect people against smallpox (Théodoridès, 1979). This author also reported that, through an English friend, Rabaut-Pommier communicated his findings to an English physician, who promised to inform Jenner. In addition, they are a number of advances in smallpox research that predate those described above; Fewster, a colleague and friend of Jenner, described in 1768 that a previous infection with cowpox virus induced resistance to smallpox, while Jobst Bose (from Göttingen, Germany) is reported to have injected himself with cowpox five years before Jesty inoculated his family (Thurston and Williams, 2015). In fact, 300 years ago, Lady Mary Wortley Montagu, brought from Constantinople to England in 1721 a technique known as 'engraftment' against smallpox. Furthermore, it appears that Chinese practitioners had already developed a variolation technique in the fifteenth century, based on inhalation of powdered smallpox material; hence, it is likely that Marco Polo brought this procedure to Constantinople. Smallpox is believed to have affected humanity for at least 3,000 years, as suggested by examination of Egyptian mummies, although some people speculate that this disease started much earlier, in the early agricultural settlements, around 12,000 years ago; this illness was described as "the most dreadful scourge of the human species" (Fenner, 1984). Smallpox became endemic disease that displayed a high mortality rate, it was estimated to cause as many as 300 million deaths, just in the 20th century. This is an illness that played a major role in history, in particular it is estimated to have killed 90% of Native Americans; the disease reached the American continent in 1520, carried by an infected African slave transported from Cuba. The social pressure to control smallpox was so strong in the late 18th century that Charles IV (King of Spain and the Spanish Empire) organized a 'Royal Philanthropic Vaccine Expedition', known as the "Balmis Expedition", that set off from La Coruña (Spain) in the corvette "María Pita". Between 1803 and 1806, the Spanish doctor Francisco Javier de Balmis led the health expedition, which vaccinated

millions of inhabitants in the Spanish territories both in the Americas and in Asia. Twenty-five orphan boys (aged 3 to 10) were required to carry the cowpox virus used in the vaccination (2 children were inoculated at a time, as it was the only known way to transport the mild virus) throughout the expedition; the mission was a success, as described in the Canary Islands (Spain, the first port of call), Venezuela, Colombia, Ecuador, Peru, the Philippines, and China. Some reports indicate that Dr John Clinch may have been vaccinating people in Newfoundland, at least one year before Jenner´s 1800 publication (McIntyre and Houston, 1999).

Although it started with smallpox (a virus), the principle of attenuation has been widely applied by microbiologists and virologists. The British Medical Journal dedicated a large portion of its publication, on the 29th of December 1883, to advances in the attenuation of human pathogens; it included reports by a variety of researchers, such as Fehleisen about erysipelas, Friedländer concerning pneumonia, Pasteur regarding rabies (Hunt, 1881), Koch and Pasteur about anthrax, and Pasteur concerning avian cholera. Hence, the attenuation studies included several bacterial species, in particular *Streptococcus pyogenes* (group A β-hemolytic streptococci), *Streptococcus pneumoniae* and *Bacillus anthracis*, not just viruses, such as the *Rabies lyssavirus.* These early reports were based on the observation that, passage of pathogens through different animals (such as sheep and oxen), or in culture media, as well as treatment of microorganisms at elevated temperatures, generated new strains with a lower pathogenicity index. The lower virulence displayed by the novel strains generated could, in some cases, be associated to the loss of a component in the pathogen, such as the capsule or another cellular structure, but, in most cases, it remained unexplained; fortunately, all the attenuated strains displayed virulence and, hence, had the potential of being used as safe vaccines.

The concept of virulence attenuation is applicable to both, diseases that infect people once and produce lifetime immunity (such as yellow fever, smallpox, typhus or measles), and illnesses (for example cholera or diphtheria) that induce immunity for only a few years, or even just a few months; the latter can infect people multiple times in their life, although subsequent diseases are usually milder.

The present chapter will focus on bacterial virulence attenuation, and does not include viruses, fungi or protozoa.

1. Bacterial virulence attenuation

Although the term "vaccination" originated from the cowpox virus 'vaccinia' (derived from the Latin word 'vacca', meaning cow), this denomination is currently accepted to indicate treatment with a vaccine, that provides immunity against a particular disease; a vaccine is a preparation that contains either whole microorganisms (attenuated or dead) or fractions of them, such as immunologically relevant molecules. According to the Oxford English Dictionary, the term vaccine was coined in 1800, and vaccination in 1803; before the use of the word vaccination became widespread, people used terms such as inoculation, attenuation and neutralization, to indicate different techniques and manipulations aimed to provide protection against diseases (Willoughby, 1884, 1885, 1888). At some stage in the past, until the theory was refuted by Moore in 1891, high altitude was recommended in the treatment of certain diseases, as it was believed that it produced microbial attenuation; this was part of the treatment for patients suffering from tuberculosis (known at the time as 'phthisis'), as some physician believed that high altitude rendered people less susceptible to phthisis. Willoughby published an article, in the last quarter of the 19th century, that summarizes the concept of immunization at that time: "The idea which underlies all protective inoculation, at least all hitherto practised, is the induction of the particular disease in a form so mild or so modified as not to endanger life, yet sufficiently defined as to confer an immunity similar, if not equal, to that which follows an attack of the disease incurred in the ordinary way". This definition of vaccination/attenuation is still current, except perhaps for the fact that people these days, due to the great improvements in health care and life expectancy, are no longer aware of the devastating effects of many diseases, and fear instead the side effects of vaccination; hence, the aim currently is for the vaccines not to produce any side effects or, at least, either minimize the risk of side effects or generate very mild reactions.

Since the second half of the 19th century, after the period denominated "the golden age of Microbiology" (in the 1850s), the scientific community accumulated a wealth of information on attenuation. Along these lines is the research carried out by Pasteur, who described the attenuation effect of oxygen on the pathogen *Pasteurella multocida*, that causes fowl cholera, as well as the inability of *Bacillus anthracis* to sporulate at temperatures ranging from 42 to 43ºC; *B. anthracis* (that produces anthrax in animals) losses its ability to sporulate after incubation for 8 days at those temperatures, making the non-sporulating bacteria good candidates for use in protective inoculations. Similarly, Toussaint demonstrated that, if the

anthrax bacterium was incubated, for a short time, at a temperature just below lethal conditions, it generated a bacterial strain with reduced virulence, hence, producing attenuated bacteria that could be used to inoculate animals and protect them against the pathogen; Toussaint also investigated the effect of certain antiseptic agents (most notably sodium hyposulphite and ethanol) on pathogens, an approach that also resulted in the production of attenuated strains that could be used as vaccines (Sternberg, 1892). Virulence attenuation was a major topic of interest at the time, as by Sir Joseph Lister eloquently described in his speech, at the University of the Sorbonne (Paris, France) on the 31st of December of 1892, to honor Pasteur on his 70th birthday. Lister was invited to give the address, congratulating Pasteur for his lifetime achievements, after dedicating his entire life to the study of pathogens and how to prevent or cure diseases; what follows is a sentence in Dr. Lister´s speech that summarizes Pasteur's research in virulence attenuation: "In this path your beautiful discoveries as to the attenuation and intensification of viruses and preventive inoculations serve, and will always serve, as a guiding star" (Br Med J, 1892).

It was mostly accepted, by both microbiologists and physicians that, those microorganisms that produce chronic diseases, such as *Mycobacterium tuberculosis*, are more likely to undergo attenuation, due to long-term interaction (up to several months) with the human immune system (Smith, 1898). A few years later, Smith (1905) described that, when two closely relate mycobacteria, *Mycobacterium bovis* and *M. tuberculosis*, were subjected to serial passage through rabbits, they reacted in opposite manners to attenuation signals produced by the animals; while *M. bovis* increased its virulence, *M. tuberculosis* underwent virulence attenuation. The studies on bacterial attenuation continued during those early years, for example, Hale and Melia, in 1910, analyzed the effect of bile on the attenuation of *Bacterium coli* (reclassified as *Escherichia coli*), while Noguchi, in 1914, and Rowland in the same year reported on the attenuating effect of fresh serum when added to microbial pathogens. In 1922, William A. Hagan published an article, based on a previous publication by Smith and Fabyan of 1912, on *Bacillus abortus* (reclassified as *Brucella abortus*), that induces abortions/stillbirths in pregnant guinea pigs; the author indirectly demonstrated that, virulence attenuation in this pathogen is not simply due to serial *in vitro* passages, but also depends on whether the bacterial culture is fresh and on the amount of pathogen inoculated into the animal.

Unfortunately, many of the attenuated bacterial strains have the ability to revert their virulence status, regressing to the 'wild type' under certain circumstances, and may even, acquire a higher pathogenic potential; to the best of our knowledge, Felton and Dougherty were the first to publish this information in 1924, based on their studies in *Streptococcus pneumoniae.*

The etiological agent of human phthisis was isolated by Koch in 1882, although, both this author and Albert Calmette, believed that the same pathogen caused human and bovine tuberculosis; it was only in 1895 that Theobald Smith realized that the two illnesses were produced by different *Mycobacterium* species, a fact recognized by Koch in 1901. Although many researchers attempted to obtain virulence attenuated strains for both species, the most successful were Albert Calmette and Camile Guerin, who managed this by sub-culturing *Mycobacterium bovis* over 200 times, during a period of almost 13 years, on a glycerin-bile-potato mixture (Calmette et al., 1924); for more information on the Bacillus Calmette-Guérin (BCG), please see the review by Luca and Mihaescu in 2013. Despite multitude of attempts to control phthisis with the appropriate chemicals, according to Paul Ehrlich's 'magic bullet' theory (stating that chemicals could be specifically designed to bind and kill particular microorganisms or cells; making Ehrlich the founder of chemotherapy), by 1920 scientists had to reluctantly admit that their efforts should be turned towards the design of vaccines that provided immunity against the pathogens. It was finally Nathan Raw who, in 1921, managed to produce attenuated strains of both *M. tuberculosis* and *M. bovis*; Raw obtained the non-attenuated bacterium that causes human tuberculosis from Koch (a bacterial strain affecting primarily lungs, generating tuberculous laryngitis and secondary intestinal ulceration), while Calmette provided the non-attenuated *M. bovis* (it produced mesenteric tuberculosis, surgical tuberculosis of bones and joints and, frequently, tuberculous meningitis). It took Raw 14 years to obtain the virulence attenuated *Mycobacterium* strains, he started subculturing the microorganisms in 1906 and, after 184 bacterial passages (only interrupted by WWI), he generated strains of both mycobacteria that were alive but avirulent. In fact, the attenuated strains did not produce either of the mycobacterial diseases; Raw summarized his achievement with the following words: "We have in our hands a remedy against tuberculosis which will be of the greatest value, not only in the cure of the disease, but what is of still greater importance, in its prevention". This is quite an accomplishment, taking into account that, as late as 1905, Calmette and Guerin believed that pulmonary tuberculosis was acquired in the early stages of life, via the intestinal route (usually from the ingestion contaminated cow´s milk), and that a single

Mycobacterium species was responsible for both human and cattle tuberculosis (The British Medical Journal, December 23rd, 1905). Also in 1921, Theodore Shennan published an article suggesting that, the same approach used by Calmette to develop a vaccine to protect cattle against tuberculosis, could be applied to produce a vaccine against human tuberculosis. In conclusion, during the pioneering years of vaccine development, bacterial virulence attenuation provided the means to generate many bacterial strains suitable for use in vaccination; currently, the aim of bacterial attenuation remains the same, to obtain non-virulent strains of pathogenic bacteria, that cannot revert towards pathogenicity, in order to manufacture vaccines to protect both humans (human medicine) and animals (veterinary medicine) against a given disease, with the condition that they must do this either without or with minimal side effects. In addition, these vaccines based on bacterial attenuation opened the way to a further development, involving the use of bacteria as carriers for foreign genes, that can be inserted into the bacterial genome and provide protection against additional diseases.

The phylogenetic relationship between humans and animals accounts for the similarities between animal and human infectious diseases, concerning the signs and side effects of the illnesses, but the ultimate goal is different in both groups (Meeusen et al., 2007; Lee et al., 2012); unfortunately, in a world centered on humans, the objective of animal care does not necessarily centers on animal welfare, but often relates to increasing animal productivity, or other factors concerning human interests. As indicated by Lee and colleagues (2012), even when large vaccination plans are developed for wild animals, rather than protect wildlife, the aim is usually to stop the spread of potentially dangerous zoonotic diseases, which threaten humans or their domestic animals, either those of economic interest or companion animals. It is worth pointing out here the danger of past mistakes, involving the misuse and overuse of anti-biotherapy in animals, that has created major problems, such a worldwide increase in antimicrobial resistance; many countries currently ban these approaches, establishing strict standards for human and animal safety (Casewell et al., 2003; Kuehn, 2012; Lee et al., 2012). As indicated by Lee and co-workers in 2012, animal vaccine development is far behind its human counterpart (Meeusen et al., 2007), but they are cheaper to produce, since they require lower quality controls. This is an area that needs urgent progress, as vaccinating the animals not only curtails the propagation of zoonotic infections, but also prevents human illness; zoonotic diseases have caused human pandemics all through our history (i. e. the bacterium *Yersinia pestis*, transmitted by fleas, that causes the ‘Black Death’, an illness

estimated to have killed 25 million people in the 14th century in Europe, almost a third of the European population at the time) and, even currently, these diseases can cause havoc around the world, as is the case for COVID-19 and avian influenza, two zoonotic viral diseases that cause millions of deaths worldwide.

Traditionally, (Fig. 1) the vaccines used in both human and veterinary medicine are classified into four groups: i) live attenuated vaccines (LAV), such as the BCG mentioned above, ii) inactivated or killed vaccines, where the cell physical unity remains almost intact, but the organism is killed by harsh treatments, such as high temperature or formalin, this is the case the pertussis vaccine (inactivated strain of *Bordetella pertussis*, etiological agent of whooping cough), iii) subunit vaccine, containing an antigen purified from the pathogen, as is the case for *Haemophilus influenzae* type b (Hib) and the pneumococcal conjugate vaccine (PCV-7, PCV-10, PCV-13), and iv) inactivated toxins (toxoids), such as the tetanus toxoid (TT) and the diphtheria toxoid (DT). The vaccine most commonly used around the world, to protect infants against Diphtheria, Tetanus and Pertussis, is the trivalent vaccine DTP, that combines approaches ii) and iv). In veterinary medicine, live attenuated vaccines are predominant, sometimes containing a modification, known as 'marker vaccine', which allows for immunological differentiation of vaccinated versus infected animals (van Oirschot et al., 1986); for ethical reasons, to the best of our knowledge, this type of vaccine has not been administered to humans. An additional type of vaccines use 'gene knockout technology' to remove the bacterial genes responsible for virulence; this approach requires a considerable genetic knowledge of the microorganism, but produces safe attenuated vaccines without the requirement for extensive bacterial passage (Xiong et al., 2017)

All the vaccine types described above have advantages and disadvantages; for example, LAVs induce high and long lasting immune responses, but they can contribute to the production of CD8+ cytotoxic T lymphocytes and T-dependent antibody responses, hence, causing side effects that can even be comparable to those produced by the untreated pathogen (Badgett, 2002). The attenuated strains provided by LAV, continue their growth in the vaccine recipients, providing a continuous supply of antigens and, hence, facilitating the development and differentiation of B and T lymphocytes, provoking a protective immune response. It must be noted that, although it is rare, in some cases LAV can revert to its full pathogenic phenotype; this is a drawback that inactivated or killed (type ii) vaccines do not have. In any case, vaccination with LAV is only recommended in healthy patients, never in immune compromised people;

another downside of this type of vaccines is that, in the case of LAVs that produce a strong immune response (such as BCG), repetitive inoculations can cause hyper-stimulation, which can even lead to lymphadenitis. On the positive side, LAVs only require one or two doses to be effective (Vetter et al., 2018) and they quickly generate an immunological response, in addition to being economical to produce (Minor, 2015). Current vaccinations that use the LAV technique include anthrax, cholera, plague, salmonella, tuberculosis, typhoid, and enterotoxigenic *Escherichia coli*.

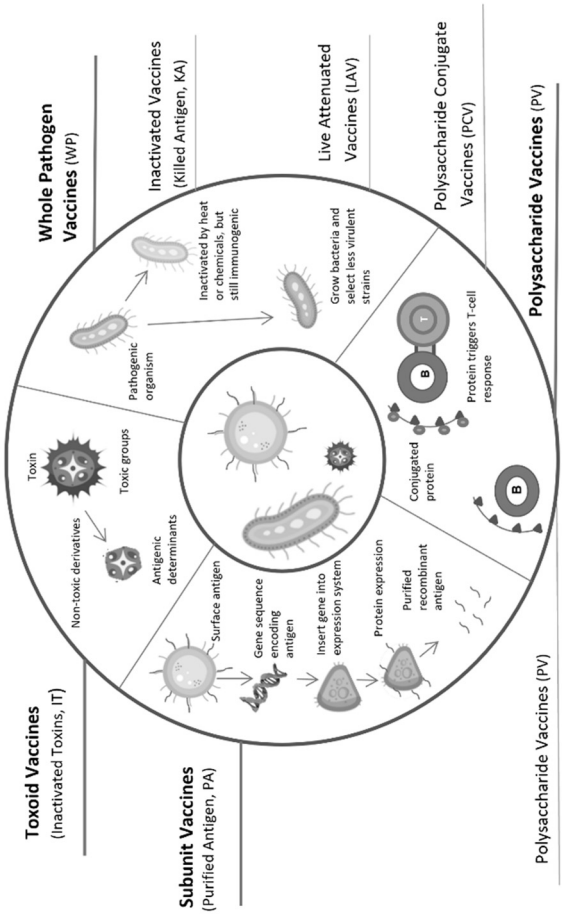

Fig.1 A summary of the types of vaccines currently in use.

An interesting feature of LAVs is that, apart from providing immunity against the diseases they are targeted to, in some cases, vaccination can produce additional, unexpected, positive outcomes; one such examples is the BCG vaccine, possibly the paradigm of LAV, which has been in use for over 100 years to combat tuberculosis. Apart from its main function, this vaccine is also considered as a form of immunotherapy in the treatment of bladder cancer (estimated 90% success rate), conferring also a lower risk of developing lung cancers, and improving the utilization of sugar by diabetics; there have also been reports indicating that BCG vaccination reduces the severity of COVID-19, this is currently being tested by the 'BRACE' trial, that is trying to determine the effect of this vaccine on the incidence and severity of COVID-19. The explanation for this protective activity appears to reside on the fact that the vaccine causes the development of heterologous immunity (an immune response directed to a new infectious agent after exposure to, or vaccination with, an unrelated pathogen), which affects CD4 and CD8 memory cell types, and increases cytokine production; in fact, Freund´s adjuvant, a solution routinely used as an immunopotentiator in experimental immunizations, is based on BCG components. In addition, the BCG vaccine can also produce epigenetic reprogramming of monocytes, by increasing histone methylation; these epigenetic changes, together with the increased production of cytokines, mentioned above, is what is known as 'trained immunity' (Bagheri and Montazeri, 2021).

As mentioned above, the traditional attenuation methods, involving the use of chemicals or long term cultivation, involving countless passages either in the laboratory or in animals, generate many non-virulent bacterial strains that have not undergone genetic characterization, a time-consuming procedure, which, in addition, could revert to their original virulent state.

This conventional approach to attenuation can only be used to produce vaccines against homologous pathogens, this is pathogenic strains either belonging to the same species or to closely related bacterial species; in some rare cases, this technique can also be used for bacteria that belong to different genera. To expand the use of the attenuated bacterial strains available, a strategy was designed to use the microorganisms as Trojan horses, to deliver foreign antigens to vaccinate against other pathogens; this is also facilitated by the fact that many bacteria can infect via the mucosal route, making heterologous vaccines easy to deliver. Some bacteria are intracellular pathogens, located inside the human or animal cells they infect, where the circulating antibodies, from the humoral response, cannot reach then; this is the case for *M. tuberculosis, M. leprae,*

Listeria monocytogenes, and *Francisella tularensis*. These particular infectious agents would require an intracellular attenuated pathogen as carrier, to safely deliver the antigen without disease generation (Mollenkopf et al., 2001). According to Roland and colleagues in 2005, most of the progress in this area was achieved using mutant strains of *Salmonella enterica*, serovars Typhi and Thyphimurium, as well as of *Vibrio cholerae*, *Shigella flexneri* and *L. monocytogenes*; these bacterial species have a natural affinity towards animal tissues, which facilitates the precise delivery of the foreign antigens, both homologous and heterologous, carried by the attenuated *S. typhi* used as a vector. This approach was used to engineer a successful oral vaccine against HBV (hepatitis B virus); an attenuated strain of *S. typhi* served as a vector, while the HBV core (capsid) protein was the foreign antigen (Schödel et al., 1994). Additional examples include the use of a non-virulent strain of *S. typhimurium*, harboring the cytidine deaminase gene as the foreign antigen, as an intravenous vaccine targeting tumors (Low et al., 1999; Zhou et al., 2018), and the design of an attenuated strain of *M. bovis* (BCG), harboring the mycobacterial antigen 85 (Ag85) complex, to combat tuberculosis (Harth et al., 2004). Recently, Ag85 was engineered, by Babaki and co-workers in 2017, for use, not just in vaccine preparation, but also as a diagnostic tool. Other applications involved the use of attenuated strains of microorganisms that did not contain foreign antigens, such as *L. monocytogenes*, to prepare an oral vaccine against listeriosis (Angelakopoulos et al., 2002), and *Shigella dysenteriae* to combat bacterial dysentery (Kotloff et al., 2002).

More recent attenuation approaches include genetic techniques, such as ‘gene knockout’; this procedure has the advantage of generating strains containing specific genetic modifications. Although the term ‘knockout’ originates from boxing, it is applied in Molecular Biology to indicate a gene that has been permanently modified, in a way that it can no longer express a functional protein; knocking out a gene does not necessarily mean removing the sequence from the bacterial genome, it can just mean that the gene cannot produce the protein, or even that the expressed polypeptide is not functional. On the other hand, the term knock-in involves the genetic engineered insertion of a new gene, a technique that can be applied to both virulence attenuation and in the design of new vaccines, as the bacteria can express a foreign gene (Husseiny and Hensel, 2005).

The gene knockout technique probably represents the most direct way to study the biological function of a given gene; this method was developed in the late 1980s, as a result of the tenacious work of Capecci (1989). The

approach was initially designed to generate knockout mice, and required a homology between two gene alleles that extended for at least 2,000 bp; this technique, however, can also be applied to any organism in which homologous recombination occurs between two quasi-equal DNA fragments. The gene knockout procedure constitutes a precise approach to obtain attenuated bacterial strains that cannot revert their virulence; the first step consists on identifying the gene involved in virulence, and this is followed by mutating the gene *in vitro*, so it no longer encodes a functional protein. The bacterium is then transfected, by electroporation, with the mutated gene, and the colonies harboring it selected, this is normally assessed by polymerase chain reaction (PCR). The bacterial strains containing the mutated gene are expected to lack the virulence factor, hence represent attenuated bacteria. Nevertheless, the lack of virulence cannot be assumed, so attenuation must be verified in animals. Another approach to gene targeting involves the use of site specific nucleases, which generate double-stranded breaks in the bacterial DNA; ligation of non-homologous DNA ends causes shifts in the reading frame, altering the resulting protein sequence, and producing a non-functional mutated gene (Santiago et al., 2008). Additional gene targeting approaches, include the use of zinc finger nucleases (artificially-generated restriction enzymes that contain a zinc finger domain fused to a DNA-cleavage domain), or the CRISPR technology. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) refers to a group of related DNA sequences, found in *Bacteria* and *Archaea*, that can recognize and alter specific DNA fragments; it was originally described, and named, by Francisco Mojica in 1993, who later speculated that it was part of a microbial immune defense (Mojica et al., 2009). When CRISPR was commercialized, as a system that could be engineered to cut specific DNA sequences, Mojica's role was all but forgotten in favor of the people who developed the gene editing technique; the CRISPR technology has revolutionized gene editing, making it precise, simpler and cheaper, it is currently applied in a great variety research fields and has the potential to be used in any organism.

Bacterial physiological responses can also be exploited to obtain novel attenuated microbial strains. A look at the history of human pathogens, and *S. pneumoniae* in particular, confirms that, in the past (Villa et al, 2019), there were pathogenic strains with lower capacity to cause the primary typical pneumonia in people; the reason for the lower virulence of these pathogens is the lack, of reduced production, of a peptidic hormone known as the 'transformation principle'. This hormone of 5-10 kDa allows *S. pneumoniae* to uptake single stranded DNA (ssDNA), both homologous

and heterologous; in fact, this probably represented the first discovery of a 'quorum sensing' response in bacteria (several 'quorum sensing' responses can occur in the same bacterial species, Fong et al., 2018). This mechanism facilitates the gathering of individual microbial cells, to reach a critical population number, when a critical mass is required to achieve the task at hand; in this case, the lower limit essential to produce pneumonia. There are additional genes that are essential for bacterial virulence, and their deletion or mutation impacts bacterial virulence, thus generating attenuated strains; that is the case for *S. pneumoniae* virulence factor HtrA, since its deletion abolishes virulence (Ibrahim et al., 2004). HtrA (high-temperature requirement A, also referred to as DegP or DO protease; Seol et al., 1991) is a highly conserved factor in bacteria, that belongs to the group of heat-shock proteins. Lanie and colleagues published in 2007 a very interesting article, analyzing the genomic sequences of two different *S. pneumoniae* strains, where the parental strain, D39, is fully virulent, while is R6 avirulent; these microorganisms are well known in biology for a variety of reasons, including the fact that they were used by Avery and co-workers (1944) to demonstrate that DNA is the molecule where heredity resides. Lanie and coworkers compared R6 to the parental strain, and discovered that the avirulent R6 contained naturally occurring mutations, that included 71 base changes, six deletions and four regions spanning 71 single-base-pair changes.

Another type of quorum sensing mechanisms, present in Gram-negative bacteria, uses autoinducers, that are signaling molecules such as N-acylated L-homoserine lactone (AHL) (Fuqua and Greenberg, 2002; Williams et al., 2007), and their cognate receptors (Joint et al, 2007; Palmer et al., 2011) in order to reach the critical population number required. *Vibrio fischeri* is the paradigm of this mechanisms, a marine rod-shaped bacterium with luminescent properties, that represents the first Gram-negative bacteria where quorum sensing was described; this microorganism controls its bioluminescence by excreting the autoinducer molecule N-(3-oxo)-hexanoyl L-homoserine lactone, that interacts with LuxR and activates the luminescence (lux) genes (Nealson et al., 1970). Once it was understood how this quorum sensing mechanism works, it became possible to engineer it to modulate bacterial virulence; the idea is to use a synthetic modulator that would stop the quorum sensing mechanism, hence inhibiting the bacterial gathering response, and preventing the microorganism from reaching the critical mass required to trigger the disease. Under these conditions, pathogenic bacteria would behave as attenuated strains, with the advantage that they would still possess all of their bacterial antigens, as this type of attenuation only

requires losing the ability to use the quorum sensing communication mechanism. This approach was first applied by Palmer and co-workers, in 2011, to successfully prevent a plant bacterial pathogen (*Pectobacterium carotovora*) from infecting either potato (*Solanum tuberosum*) on green bean *(Phaseolus vulgaris*) plants; the idea was to trial the system under native conditions, in a natural environment. This successful application should be the catalyst for future research into the use of this technique to prevent bacterial infections in animals, and even people. In fact, the way was paved by Tateda and colleagues, who in 2004 reported that certain macrolides act as suppressors of the quorum sensing response is *Pseudomonas aeruginosa*; this human pathogen is involved in the development of cystic fibrosis and diffuse panbronchiolitis. More recent publications add to the list of compounds that could be used as quorum sensing inhibitors, these include coumarin, that inhibits this communication mechanism in *P. aeruginosa*, as well as impeding luminescence production in *V. fischeri* and many other bacteria, hence indicating that this approach could have a wide-ranging efficacy in combination with current antibiotics (Gutiérrez-Barranquero et al., 2015). Accordingly, Paczkowski and colleagues demonstrated, in 2017, that the virulence of *P. aeruginosa* could be attenuated by treatment with flavonoids, a type of plant pigments that allosterically inhibit the quorum sensing receptors. Fong suggested a combination of two approaches, the use of inhibitors together with quorum quenching enzymes, such as the quorum-quenching N-acyl-homoserine lactonase (EC 3.1.1.81, quorum-quenching N-acyl homoserine lactone hydrolase; Dong et al., 2000), in bacterial species, including *P. aeruginosa*, that contain multiple quorum sensing systems (Fong et al., 2018). In 2019, Xu and coworkers reported that the organosulfur compound allicin (found in garlic and originally isolated and studied by Chester Cavallito and John Hays Bailey in 1944) inhibits *P. aeruginosa* virulence, by interfering with the quorum sensing pathways (Xu et al., 2019).

Under harsh conditions that cannot support proper growth, both bacteria and plants, can synthesize special GTP/GDP-derived molecules, collectively known as ‘alarmones’ (ppGpp and pppGpp); these are intracellular signaling molecules that are produced when the organism is under stress, for example, in the case of amino acid depletion, or when bacteria are treated with antibiotics, in this situations, the alarmone levels can increase considerably, 100 times over normal concentrations (Fernández-Coll and Cashel, 2020). When *Enterococcus faecalis* is treated with vancomycin, alarmone production induces a phenotype of antibiotic tolerance (Abranches et al., 2009). These signaling molecules were

originally described by Cashel and Gallant in 1969, and the stress signaling pathway was denominated ‘stringent control’ or ‘stringent response’ (Cashel and Rudd, 1987); the alarmones bind to the β and β′ subunits of the DNA dependent RNA polymerase (EC 2.7.7.6), halting most of the protein transcription, and the bacterial growth, with only a handful of polypeptides produced. The rationale behind this approach is to control the metabolism of these alarmones, or ‘magic spots’ (Potrykus and Cashel, 2008) as they were originally nominated, to obtain attenuated bacterial strains, that still contain all their immunological potential. According to Dalebroux and colleagues (2010), two enzymes, RelA and SpoT Homologue (RSH), synthesize these transcriptional modulators; RelA is monofunctional polypeptide that only acts as a synthetase, while SpoT Homologue is a bifunctional protein, working either as a synthetase or a hydrolase (Magnusson et al., 2005; Srivatsan and Wang, 2008). Most of the gammaproteobacteria species contain the corresponding genes for RelA and SpoT, while mycobacteria, alphaproteobacteria and epsilonproteobacteria only contain a single bifunctional RSH protein; microorganisms belonging to the phylum *Firmicutes* encode, apart from the bifunctional RSH protein, a RelA-like synthetase, while *V. cholera* contains an additional protein, RelV, that represents a novel synthetase enzyme (Das et al., 2009). As concluded by Dalebroux and co-workers (2010), the fact that bacteria invest so many genes and energy into this stress response, must indicate that the alarmones are well balanced for a proper bacterial homeostasis growth; if, for example, the hydrolase activity gene is mutated, the hyperaccumulation of these typical nucleotides would result in a stop in growth, this is of particular importance for intracellular pathogens, in which SpoT directs bacterial differentiation inside the macrophages (Dalebroux et al., 2009; Gaca et al., 2015). All the information currently available indicates that these alarmones control bacterial virulence, hence, controlling these signaling metabolites, via engineering the key enzymes involved, would have a major effect on the virulence and, hence, pathogenic status of these microorganisms (Kundra et al., 2020); this approach would also result in the generation of novel attenuated bacterial strains, or, as indicated by Gaca and co-workers in 2015, would eventually provide a mechanism to control long-term bacterial survival strategies. Indeed, some nucleotides are involved in translation quality control, ensuring that the proper responses to stress are implemented and that translation is accurately conducted (Bullwinkle and Ibba, 2016); as it is well known, translation fidelity is accomplished through the correct tRNA–codon pairing within the ribosome, as well as by assuring that each amino acid is properly

attached to its corresponding tRNA, through the action of their respective aminoacyl-tRNA synthetases. In summary, the fidelity of translation rests on the ability of the enzymes, involved in the process, to distinguish between cognate and non-cognate amino acids (Yadavalli and Ibba, 2012; Bullwinkle and Ibba, 2016). It was recently reported that, when bacteria enter quiescence, the alarmones can directly inhibit translation initiation, by binding to the essential GTPase Initiation factor 2 (IF2) (Diez et al., 2020).

Nelson and Breaker (2017) described a novel universal alarmone, named ZTP (5-amino 4-imidazole carboxamide riboside 5'-triphosphate), also known as the ZMP/ZTP riboswitch; the latter refers to a conserved RNA structure found in some bacteria (Weinberg et al., 2010), that controls zinc (Zn) concentration in sporulating Gram-positive microorganisms, such as *B. subtilis* (Nies, 2019). Zinc ions are redox inert, and this metal is essential for appropriate cell homeostasis, albeit in very small amounts (*ca*. $70x10^3$ ions/cell; Outten and O'Halloran, 2001; Nies et al., 2019), as high levels of Zn are toxic for the cells (Andreini et al., 2006); zinc acts as a molecular chaperone, and is a crucial component of essential enzymes, such as DNA-dependent RNA polymerase, being also involved in the assembly of its subunits (by acting on the β subunit) during the initiation of the transcriptional process (Markov et al., 1999). Zinc ions are also play a role in the *de novo* synthesis of folate, a crucial process in bacteria (Sankaran et al., 2009). These data, taken together, indicates that controlling the intracellular levels of zinc represents a way to control the growth of certain bacteria, and may even constitute an alternative pathway to obtain novel attenuated microbial strains.

The use of bacteriophages to obtain bacterial attenuated strains is a relatively recent concept in vaccine manufacture. In 2010 Capparelli and colleagues reported that *Staphylococcus aureus* strain A172, resistant to infection by phage MSa, produced low amounts of capsular polysaccharide; A172 was also described to regulate the transcription of cytokine genes, such as TNF-α, IFN-γ and IL-1β, and to behave as an attenuated strain, independently of whether it was alive or heat killed, when administered either intramuscularly or through aerosols. In addition, the authors described that the heat killed strain provided supplementary advantages when used as a vaccine, at least in mice, as it protected the animals against methicillin-resistant *Staphylococcus* strains. Filippov and co-workers (2011) obtained similar results with *Yersinia pestis*, the authors identified phage-resistant mutants of this pathogen that behaved as attenuated strains in mice. A year later, Laanto et al. (2012) reported that

in *Flavobacterium columnare* (a pathogenic bacterium significant in aquaculture), resistance to bacteriophages was also associated with loss of virulence, generating organisms that behaved as attenuated strains. Furthermore, Castillo and co-workers (2015) discovered that this was also true for *Flavobacterium psychrophilum*, a pathogen in salmonid farms. These results, together with the findings of Shen and Loessner (2021), who described phages as modulators of bacterial virulence, open novel possibilities for the use of bacteriophages in human and animal therapy; as phages only infect bacteria, they could be used to combat bacterial pathogens infecting humans or animals. The viruses would kill most of the microorganisms, with only the bacteriophage resistant strains surviving; the latter representing attenuated microorganism that, apart from not producing disease, would generate immunity against the illness.

These attenuated bacterial pathogens could also be used as carriers to deliver foreign antigens, or even heterologous DNA (if/when DNA vaccines are permitted for use in humans and/or animals), and could constitute important vaccine vessels (Yurina, 2018). Following are several examples of attenuation of classical bacterial pathogens, that still currently threaten humanity.

Table 1 summarizes the main properties of the bacterial toxins produced by these pathogens, while Fig. 2 compiles important events in vaccine development.

Table 1 Summary of relevant characteristics displayed by the bacterial toxins reviewed in this chapter

Toxin	Bacteria	Structure	Target	Receptor	Activity	Effects	Reference
Tetanus neurotoxin	*Clostridium tetani*	Single chain protein (150 kDa)	Inhibitory interneuron gangliosides (GD1b, GT1b)	GPI-anchored protein	Proteolysis of SNARE protein (VAMP)	Inhibition of neurotransmitter release (GABA, glycine), spastic paralysis	Popoff and Poulain, 2010
Cholera toxin	*Vibrio cholerae*	AB structure	Enterochromaffin cells and enteric neurons, G-protein	Ganglioside GM1	Inactivation of Gsα and activation of adenylate cyclase 5-HT	Rice-like, watery diarrhea. High volume depositions	Popoff and Poulain, 2010.
Shiga toxin	*Shigella*	AB structure	rRNA (28S)	Gb3 glycolipid	N-glycosidase, cleaves adenine 4324	Bloody diarrhea and hemolytic uremic syndrome	Odumosu et al., 2010.
Pertussis toxin	*Bordetella pertussis*	S1-S5	G-protein	Various gangliosides	ADP-ribosyl transferase	Lymphocytosis and alteration of hormonal activities regulated by Camp	Odumosu et al., 2010.

Anthrax toxin	*Bacillus anthracis*	Protective antigen (PA), lethal factor (LF) and edema factor (EF)	MAPKK	ANTXR 1	Zn metalloprotease	Endothelial cell barrier dysregulation, coagulopathy, RBC death, inhibition of neutrophil mobility, phagocytosis, and alterations in cytokine modulation. Inhibition of superoxide production by neutrophils and cytokine production by dendritic cells.	Odumosu et al., 2010.
Anthrax toxin	*Bacillus anthracis*		Protein kinases	ANTXR 2	Adenylate cyclase		Odumosu et al., 2010.
Diphteria toxin	*Corynebacterium diphteriae*	AB structure	Elongation factor (EF-2)	Elongation factor (EF-2)	Transfer of ADP-ribose from NAD to EF-2 B. Receptor-binding	Inhibition of cellular protein synthesis and cell death	Pappenheimer AM 1977.
Typhoid Toxin	*Salmonella typhi*	A2B5 structure	Immune system and central nervous system	Gangliosid e GD2, and N-glycans	ADP-ribosyl transferase (PltA), nuclease activity (CdtB) and specific glycan-binding (PltB)	Typhoid fever, Neuropsychiatric manifestations, abdominal pain, immunologic symptoms (i.e. leukopenia and neurological complications)	Chong et al., 2017.