An Overview of Alternatives for Conventional Antibiotics

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Introduction

These days, most bacterial infections are easily treated with antibiotics; however, increasing bacterial resistance against these medicines is starting to complicate treatment. We have come a long way since Alexander Fleming discovered penicillin in the late 1920s and bacterial infections were life threatening. This discovery and subsequently the development of sulphonamides for clinical use in the 1930s brought an enormous improvement of medical possibilities when their use became widespread after World War II. The availability of antibiotics has greatly increased our ability to treat serious infections, but has also opened new doors with respect to surgery, neonatal medicine and cancer treatment [1].

Since then the use of antibiotics has spread from medicine to agriculture, where they are used for treatment of disease, prophylaxis and growth promotion in animals and to treat or prevent plant diseases, both on food crops and flowers [4]. Many classes of antibiotics are used in human and veterinary medicine [2]. In terms of quantities, most of the antibiotics are used in agriculture: 2009 figures from the United States Food and Drug Administration (FDA) and the United States Government Accountability Office suggest as much as 80% of all antibiotics sold in the USA were used in food-producing animals [9,10]. The large scale use of antibiotics in all its applications has had a great impact on our prosperity, but a serious problem has since arisen. Even in the early days, resistance against antibiotics was described. In his nobel lecture in 1945, Fleming already warns against under dosage, stating that: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body" [5]. Although addition of antibiotics to feed as growth promotor for food animals was banned in the European Union, several antimicrobials that are considered important in the treatment of infections in human medicine, are still widely used for growth promotion in the USA [3].

The use of antibiotics, especially in sub-therapeutic concentrations, creates a selection pressure that favours resistant bacteria. These resistant bacteria are now found everywhere in our environment and are readily exchanged between reservoirs. For example, farm animals have become a reservoir of resistant bacteria, leading to frequent transmission of these bacteria to humans that consume contaminated food, such as meat, fish, dairy and crops contaminated by fertilisation with manure containing resistant bacteria [2]. Yet infection with these antibiotic resistant food-borne bacteria is not the only concern. Research has shown that non-human pathogens like *Enterococci* are able to pass their resistance genes to other bacteria in the human gut [3].

One of the best known antibiotic resistant bacteria is the methicillin-resistant *Staphylococcus aureus* (MRSA), which can cause serious infections both in the community and the hospital environment. MSRA is resistant to most β-lactam antibiotics and often carries resistance to other classes of antibiotics, which makes infections with MSRA difficult to treat. Resistance against vancomycin, one of the few antibiotics that can be used to treat MSRA infections, is also frequently reported [6] and illustrates the constant developing need for new antibiotics to avoid resistant bacteria becoming untreatable.

However, the development of new antibiotics has slowed down considerably after its mid 20th century peak. Most of them were discovered by empirical screening of fermentation products and chemicals for bacterial growth inhibition. Although knowledge and technology have improved since then to modernised screening methods and to rational target-based screening, only a few discoveries have made it to the clinic. Linezolid, daptomycin and retapamulin, members of a new class of antibiotics, that have been brought to market in the 2000s, but their classes were in fact described or patented in 1978, 1980 and 1952. An overview of the discovery of antibiotics is shown in figure 1. Other new products have been derived from existing antibiotics, improving spectrum, ease of use, safety or avoiding resistance mechanisms [7].

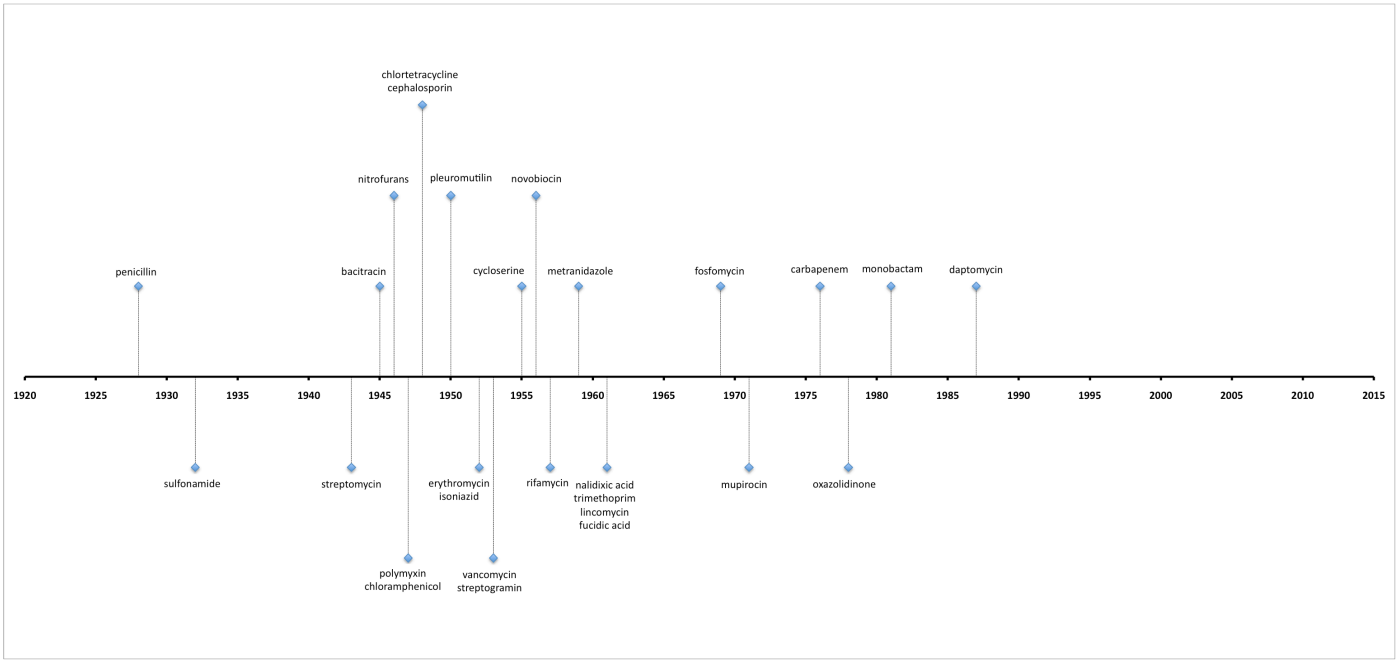

Figure 1: A timeline of the discovery of new antibiotics (adapted from [7])

There are multiple reasons for this decrease of development. In the first place, the empirical screening in the 1950s and 1960s found the 'easy' targets, making it harder to find novel antibiotics or antibiotic classes. Next, target selection comes with its own challenges: a promising target must be i) essential to the bacteria, ii) conserved within a range of species, iii) available for drugs and iv) should not resemble host structures to avoid toxicity [7]. Finally, there are significant commercial and regulatory challenges involved with the development of new antibiotics. Gaining approval for use and successfully applying for a patent is hard and costly. Furthermore the use of new antibiotics is limited because antibiotics are typically given to a patient for a short period of time and medical practitioners tend to use older medication unless resistance is suspected. Furthermore, the antibiotic's market time is uncertain due to inevitable development of resistance in bacteria [1,8].

Although the large pharmaceutical companies are investing less in the development of new antibiotics, research to find alternatives to our current antimicrobial drugs has not stopped. Scientists are still looking for alternatives to the conventional antibiotics, with many interesting angles. In this assignment I hope to give an overview of the current antimicrobial strategies, such as antimicrobial peptides, bacteriophages and probiotics, and to discuss their applications and feasibilities and the effect of combination of these methods with existing treatments.

1. Antimicrobial Peptides

Many organisms, from bacteria to plants and animals, produce antimicrobial peptides (AMPs) as a part of their defence system. These molecules come in a great variety, with a wide range of properties. They are divided based on their origin in two groups, those of prokaryotic organisms referred to as bacteriocins versus the eukaryotic host defence peptides (HDP) produced by plants and animals. They are further divided into classes based on their chemical structure. The naturally occurring peptides are seen as a promising alternative to regular antibiotics, because they are still effective defence mechanism against bacteria despite exposure throughout the centuries.

Host Defence Peptides

HDP are small (10-50 amino acids) cationic peptides. While they come in a variety of sequences and structures, they can be divided into two classes: 1) β-sheet peptides, which are stabilised by two to four disulphide bridges; 2) α-helical peptides; loop peptides with a single disulphide bridge and extended structures rich in proline, glycine, tryptophan and arginine or histidine [14]. Besides their net positive charge, the peptides have a significant amount of hydrophobic residues. These are separated within the molecule, leading to an overall amphiphilic 3D structure that explains one of the mechanisms behind their direct antimicrobial effect [14].

There are two distinct modes of action for the HDPs' activity: one targets the bacterial cell surface, the other finds its targets inside the bacterium. In the first case, HDPs are grouped at the negatively charged cell surface due to the peptides' positive charge. There they disrupt the bacterial membrane by pore formation, membrane depolarisation or disturbing the lipid composition of the membrane, which results in bacterial death. The other type of action of HDPs is to enter the bacteria, and affect various intracellular targets, thereby inhibiting essential intracellular processes needed for growth and causing cell death [12,15,16]. This shows the difference between conventional antibiotics and AMPs: while conventional antibiotics are generally targeting one primary target and have only one mode of action, AMPs often have multiple targets and combine different actions to create a bactericidal effect.

However, although many *in vitro* studies show the antimicrobial effect of HDP, the effect is often inhibited when tested in physiological conditions and their role in innate immunity might be more important when clearing bacterial infection *in vivo*. They have been shown to induce chemotaxis, suppress pro-inflammatory cytokine production induced by the bacterial products lipopolysaccharide (LPS) and lipoteichoic acid (LTA), modulate dendritic cell activation and differentiation and promote wound healing [17]. This combination of anti-infective activity and reduction of the inflammatory response could bring relief to patients and help those at risk of an excessive response, such as sepsis [13]. This way, HDP could also add value to conventional treatment in a combination therapy with regular antibiotics. This immunomodulary effect is one of the many advantages that have made these peptides such a promising alternative to conventional antibiotics.

Another is their variety: more than 1000 antimicrobial peptides have been identified by sequence [13] and there is a great diversity of structures and functions. HDP have a broad spectrum of activities, targeting both Gram-positive and Gram-negative bacteria (and in some cases fungi or viruses), including multi-drug resistant bacteria [18]. The disadvantage of the broad spectrum activity of these antimicrobial peptides is that many commensal bacteria that protect niches that could be taken by opportunist pathogens might also be affected.

Due to the presence of HDPs in the natural environment, bacteria have been exposed to them for a very long time. Some bacteria show resistance mechanisms, for instance changes in the membrane to reduce its

charge, a system to export AMPs with intracellular targets from the cell, or proteolysis [19]. It has been suggested that these adaptations are energetically costly for the bacteria and thus hard to induce in experiments [12,18], although others have argued that this view is too optimistic, that resistance against HDPs does develop readily upon repeated exposure and that therapeutic use of HDPs should therefore be carefully regulated to avoid overuse and misuse [19,20,21]. Nonetheless, no universal resistance mechanism has yet been found [12].

Cross-resistance is another area of concern related to resistance against HDPs: even when using HDPs from other organisms, there is a risk that it translates to resistance against related HDPs. In fact cross-resistance to human [22] and non-human [21] HDPs has already been shown. Therefore, with these peptides being part of our innate immune system, development of cross-resistance could be sabotaging our defence against bacteria. On the other hand, supporters of the development of HDPs to new antibiotics state that host defence peptides are only one part of the innate immune system and that experiments with animals lacking these peptides are quite healthy. [14] However, this is one of the reasons bacteriocins are considered as a safer alternative.

There are other more practical limitations to the use of HDPs as antimicrobial therapy. These peptides are sensitive to protease degradation, which poses challenges in terms of *in vivo* stability and delivery. Due to the complex modes of action of HDPs, toxicity is also a concern: because eukaryotic membranes are less susceptible, but not invulnerable to disruption by these peptides due to the lack of negatively charged lipids and presence of cholesterol at the cell surface. Both membrane targeting and the protease-related stability problems mentioned above are avoidable by designing peptides that do not target these cell membranes. [23]. However, HDPs have been shown to functionally enter eukaryotic cell. For this reason, this effect of HDPs on the eukaryotic cell should be studied alongside direct cytotoxicity [13,14]. Finally, the feasibility of HDPs as novel antibiotics is challenged by the high costs of manufacturing peptides by chemical synthesis. Unfortunately production by less expensive methods, such as using recombinant bacteria, fungi, plants or animals on a commercial scale have failed so far. This is an enormous disadvantage when comparing this option with the cheaply produced conventional antibiotics [14].

Bacteriocins

Bacteriocins are divided in four classes: class I (lantibiotics) are small (<5kDa) peptides that contain lanthionine or β-methyllanthionine: thioether linkages between modified serine or threonines with cysteines as a result of post-translational modification [26,27]; class II contains small (<10kDa) peptides that do not contain the above mentioned amino acids; class III is made up out of larger (<30kDa) proteins that are not heat-stable like the previous two classes; and class IV bacteriocins include a wider variety of proteins including cyclic peptides and the phage-tail-like high molecular weight proteins. Within class II there are subclasses based on structure and mode of action. [24]

While HDPs associate with bacteria based on membrane charge, bacteriocins have more specific targets on the bacterial surface that function as receptors, such as mannose-phosphosetransferase systems [11] and lipid II [27, 29]. Some lantibiotics kill bacteria by inhibiting cell wall synthesis by targeting lipid II, an important intermediate in cell wall biosynthesis. Other lantibiotics form pores through the barrel-stave or wedge models; class IIa peptides and some other class II bacteriocins form pores in the cell membrane [29,27] according to the barrel-stave models as well or follow the carpet model. Pore formation leads to loss of membrane potential and leakage of metabolites or other cell contents, which results in bacterial death [24]. Finally, bacteriocins can cause bacterial death by entering the cell and inhibiting DNA, RNA or protein synthesis [25]. Like HDPs' mechanisms of action, those of bacteriocins are distinctly different from the modes of action of conventional antibiotics.

One advantage of bacteriocins is the availability of bacteriocins with a broad antimicrobial spectrum and narrow-spectrum peptides. While a broad spectrum is an advantage against unidentified bacterial species, commensal bacteria are also affected by these type of antimicrobials. Therefore, a narrow spectrum antimicrobial peptide is an advantage when treating a specific infection. The availability of such bacteriocins is one of the advantages bacteriocins have over the HDP described earlier [25].

Bacteriocins have been tested for their activity against infection both *in vitro* and *in vivo* and have shown a good potency overall. Especially research into lantibiotics and thiopeptides has been carried out extensively [25]. An extensive overview of substances and research into their application in human and veterinary medicine is given by Hammami *et al.*, who have reviewed promising applications of bacteriocins for hospital-acquired infections, respiratory, skin, gastrointestinal, urogenital infections in humans and skin, gastrointestinal and systemic infections in animals [24].

One of the advantages of bacteriocins over HDPs is that bacteriocins target molecules that are more specific to prokaryotes, which decreases this risk. This is shown by the fact that bacteriocins from lactic acid bacteria in fermented food have been ingested for centuries [25]. Nisin has even been an approved safe food preservative, and used as such, for thirty years.

Bacteriocins, like HDPs, are vulnerable for proteases, and for bacteriocins bioengineering could also be the solution to improved *in vivo* stability [26]. Moreover, bioengineering could be used to improve other properties of bacteriocins: e.g. potency, target selectivity [26]. Another advantage for bacteriocins is that they are produced in bacteria. This simplifies production because recombinant bacteria could be used, rather than having to chemically synthesise peptides from amino acid building blocks. This could also benefit bacteriocins in terms of production costs compared to HDPs.

Finally, an advantage for bacteriocins might be in the delivery system. While some bacteriocins may be stable enough to be used through standard methods, there is a potentially better way to get the peptides at the site of infection. Knowing that many bacteria produce at least one bacteriocin, and that the human body is colonised with many different commensal bacteria, one can consider the possibilities of *in situ* bacteriocin production by probiotics [25,28].

Of course, bacteriocins are not the perfect solution. As with every antimicrobial, there is a risk that bacteria develop resistances after long term exposure and *in situ* production might even increase this risk, which is important to evaluate if developing such a system. A number of possible mechanisms of resistance have been found, especially to the bacteriocins that target the cell surface. Reduced receptor accessibility or reductions of receptor expression have been observed in laboratory strains; however, intracellular modifications have also been found. Mutations in the genes encoding RNA polymerase and DNA gyrase have allowed bacteria to become more resistant to bacteriocins with those targets [11,25]. Additionally, bacteriocin-producing strains have a mechanism that makes them resistant to their own bacteriocins [29]. Exchange of these resistance genes, creating immune mimicry, would also be way for bacteria to become resistant against these peptides [25].

Applications

Although not many antimicrobial peptides have reached the stage of being available on the market, there are some exceptions. The bacteriocin nisin has been approved as a food preservative in the United States and in the European Union (since 1988 and 1983, respectively after being judged safe for food use by the World Health Organisation (WHO). Bacteriocins nisin (from certain *Lactococcus lactis* subspecies) and lacticin-3147 (from *Lactococcus lactis* DPC 3147) [24] are also successfully used in products, teat seals and wipes, for the prevention of bovine mastitis [11]. Gramicidin S (from *Bacillus brevis*) and polymyxin B (from *Bacillus*

polymyxa) have been approved for clinical use by the FDA in the United States, but is limited to topical application due to toxicity [12].

Future Outlook

So far only a few antimicrobial peptides have made it onto the market. Bacteriocins nisin as a food preservative and lacticin-3147 in veterinary mastitis preventative products; gramicidin S and polymyxin B as medicines for topical use, other AMPs are currently in various stages of clinical trials [13,14]. Gaining approval for medication remains a significant hurdle. For example, pexiganan (a variant of magainin) was rejected by the FDA despite proved efficacy due to not being more effective than treatment that was already available.

So far approval of the use of AMPs in a clinical setting has been limited to topical application due to toxicity and pharmacokinetic reasons described earlier. It will require more research to solve these problems, through new discoveries or through bioengineering known peptides. Hopefully this will result in the future in antimicrobials that can be delivered in different ways to help fight internally localised as well as systemic infections. Besides overcoming these limitations, these biomolecular techniques should also be used to find improvement in terms of efficacy of the peptides and to continue the elucidation of AMPs' modes of action and bacteria's resistance mechanisms. More research is also required in the direction of HDPs' endotoxin neutralising and immunomodulary activities and how those can be used in medicine. The possibilities for bacteriocins to be produced *in situ* by probiotics and the risks using these peptides, focusing on the potential cross-resistance of bacteria to human HDPs in the innate immune system, should be looked at closely.

An added value of antimicrobial peptides is that they might be used in combination with conventional antibiotics to work in synergy [13]. Therefore, the option of using AMPs in combination with other drugs, such as conventional antibiotics, should be studied. The pore formation by the AMPs could help make bacteria more vulnerable to the antibiotics with cytoplasmic targets and HDP immunomodulary properties could help clear infection, neutralise endotoxin or suppress the negative effects of inflammation.

Finally, like nisin, other AMPs might be used in the food industry. Many bacteriocins are produced by bacteria that are considered safe for food. This makes these peptides an answer to customers' calls for products not to contain certain chemical preservatives: they could be replaced by bacteriocins. For example, those with a broad spectrum could be added to products to prevent food spoiling, in fermented food bacteriocin-producing bacteria could be used to produce the peptides *in situ* and narrow-spectrum antimicrobial peptides could be used against known pathogens in food, such as *L. monocytogenes* [11].

2. Probiotics

Another potential alternative for conventional antibiotics are probiotics, and related to that, prebiotics. The benefits of probiotics have been known throughout history: the health qualities of yogurt have been known in Asia and the Middle East for millennia [30] and in a Persian version of the bible's old testament, it was said Abraham's longevity was due to him drinking sour milk [31]. In the 19th century, Ilya Mechnikov identified the bacterium *Lactobacillus bulgaricus* (later: *Lactobacillus delbrueckii* ssp. *bulgaricus*) in Bulgarian yogurt, building on the observations of Stamen Grigorov's documentation of the yogurt's health benefits [30] and concluded they were caused by a change in the intestinal bacterial balance [31]. Using the Greek words for 'for life' (a contrast to antibiotics: 'against life'), the term "probiotic" was coined by Werner Kollath in the 1950's to describe "active substances that are essential for a healthy development of life" [30]. Over time, the definition has been adapted to include the concept of intestinal microbial balance. In 1989, Fuller's changes avoided the misleading term 'substance' and defined probiotics to be "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance" In 2003, Reid et al. refined the definition further to "live microorganisms which when administered in adequate amounts confer a health benefit on the host." [30].

Important is that probiotics are non-pathogenic to the host, resistant to components of the digestive system, such as stomach acid, are able to (temporary) colonise the host and antagonism of pathogenic bacteria. For safety reasons, they should be free of antibiotic resistance genes, or unable to transfer these genes to other bacteria [43].

Prebiotics ('before life') are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" by Gibson and Roberfroid [32]. In 2007 they redefined this to "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gut microflora that confers benefits upon host well-being and health". Furthermore they insisted that although the ingredient need not be completely indigestible, it should be sufficiently resistant to be available in significant amounts in the large intestine [33]. Compounds referred to as prebiotics include various oligosaccharides, inulin, pyrodextrins and lactulose [30]. For products containing both probiotics and prebiotics the term symbiotic is used, referring to the synergy between them [31].

Mode of Action

The activity of probiotics is specific for the various species and varies between strains. There are a number of mechanisms that are employed: competitive exclusion, production of antimicrobial compounds, immunomodulatory effects and some other actions.

Competitive exclusion is based on the notion that bacteria compete for nutrients and attachment sites [30,31,38]. If one species is present in sufficient amounts, it may prevent establishment of another in a certain niche. For example, *Lb. salivarius* UCC118 was shown to inhibit growth of *S. typhimurium* UK1 [28]. Small changes in environmental factors might change this balance [31]. This is also the principle behind prebiotics: providing the right nutrients for beneficial bacteria, thereby giving them the advantage over less desirable species.

The bacteriocins, discussed in the previous chapter, also play a part in the mechanisms by which probiotics are active. By producing bacteriocins, a producing strain might be able to create an empty space in a fully established community, while it would otherwise transiently pass [40]. Once established, the probiotic bacteria could use these antimicrobials to inhibit the growth of bacterial pathogens. For example, Guo *et al.* considered antimicrobial compound production as one of the principal selection criteria for screening

potential probiotics [35]. Another example shows that a *Lb. saliviarius* strain inhibits *L. monocytogenes* growth by bacteriocin production [28].

The third mode of action of probiotics is their ability to affect the immune system. The immune system has to tolerate the presence of the probiotic, and must be stimulated by the probiotic to clear pathogens [39]. The presence of probiotics has been shown to stimulate phagocytosis [31,38] and to modulate macrophage proliferation and cytokine production [34,38,41]. Direct feed supplementation of probiotics resulted in an increase of white blood cells and plasma immunoglobulins in broiler chickens [36].

Finally, certain probiotics may have a positive effect against disease by inhibiting some of the virulence factors of pathogens [44].

Applications

Use of probiotics started in animal feed in the 1920s [31,34], but the name only started to get used for human and animal microbial feed supplements in the 1970s [31]. The first significant evidence for probiotics was delivered in the 1960s: it was shown that *Lactobacillus* supplementation could stimulate the growth of pigs, making *Lactobacillus* supplementation a candidate for replacing antibiotics as growth promoters [34]. In aquaculture probiotics were being investigated since the late 1980s, which allowed the decrease of antibiotic use by more than 90% in the early 1990s [31].

In fact, probiotics are very interesting for the food-animal industry. For example, *Bacillus subtilis* MA139 was shown to enhance daily weight gain and feed conversion in piglets, equal to a diet supplemented with antibiotics. At the same time *Lactobacilli* in faeces was increased whilst *E. coli* counts were decreased [35]. Other studies using *Bacillus* strains have shown similar effects in terms of weight gain [34]. In poultry probiotics have been shown to increase weight gain, decrease *E. coli* counts and improve ileal morphology, which results in a larger absorptive surface and stronger gut integrity [36]. Furthermore, improved egg production and quality has been reported after supplementing laying hens' feed with probiotics [37].

Besides growth promotion, probiotics have a veterinary value in prevention and reduction of (bacterial) disease. Various bacteria have been used in aquaculture to prevent shrimp mortality by pathogenic *vibrio* strains [31]. In pigs probiotics can be used to prevent intestinal disease caused by enterotoxigenic *E. coli* (ETEC) strains, a common cause of diarrhoea [38] and reduce the risk of food-borne pathogens, such as *Salmonella* and *E. coli* [34]. *Lactobacilli* have been shown to be effective against *Salmonella* and *Campylobacter jejuni* shedding [34,37] and improves survival rates of chickens infected with *S. typhimurium* and *E. coli* [34]. In cattle colonisation by *E. coli* can be reduced by a mixture of probiotics, which increases food safety for consumers [34].

Probiotics also have interesting applications in human medicine. It is known that although we carry many bacteria in homeostasis, changes in the environment or use of antibiotics can result in a switch causing commensal bacteria to become pathogens. This happens either because the bacteria are able to access a place in which they aren't harmless, for instance in wound infection by bacteria on the skin, or because an imbalance allows bacteria counts to increase to a level that is problematic. The latter might be caused by antibiotic use for another infection, leading to diarrhoea. Probiotics have been proved to be beneficial in restoring homeostasis [39].

For example, previous antibiotic use has been shown to be a risk factor in *Clostridium difficile* infection. Although it remains controversial, there has been some evidence that suggests probiotics, in the form of *Lactobacilli* or the yeast *Saccharomyces boulardii*, have a beneficial effect in the prevention of *C. difficile* infection when supplemented during antibiotic treatment [40]. Another example is the use of *S. Boulardii* to improve

eradication rates of *Heliobacter pylori* and diminish side-effects when used in combination with conventional antibiotics [41].

Future Outlook

In summary, there are two main applications for probiotics at the moment that can decrease our use of conventional antibiotics: prevention of infection and growth promotion. By preventing infection (a primary infection or secondary as a side effect of previous antibiotic use) it prevents the need for further use of antibiotics to clear it, and probiotics used to improve the growth of food animals might be the answer to use sub-therapeutic doses of antibiotics in feed to maximise meat production. However, many results of research on this topic are still controversial, and more knowledge is needed to clarify seemingly contradictory findings. At the same time, many of the mechanisms behind these activities are unknown; more knowledge in this area might help answering the questions around the efficacy of probiotics.

Further progress might be made if therapeutic applications for probiotics are made. Examples of this would be the bacteriocin production *in situ* mentioned in the previous chapter, which would require a way for probiotics to have a lasting presence. Another suggestion is the development of 'designer probiotics', made to neutralise toxins produced by pathogens by receptor mimicry, or removing pathogens [42].

3. Bacteriophages

Bacteriophages, or phages, were discovered independently by Twort and d'Herelle in 1915 and 1917 respectively. They discovered clear spots in an agar cultures of bacteria, later referred to as Twort-d'Herelle phenomenon [30]. While Twort called it a transmissible lytic agent, d'Herelle's name, bacteriophages, is the one still used today. D'Herelle was the first who started to experiment to use bacteriophages as a therapeutic agent [45].

Phage therapy was used with varying success rates, but interest diminished when antibiotics were discovered [30,45,46], although its use has been continued in Eastern Europe, particularly in Poland and Georgia [45,46,50,51]. Since the 1980s phage therapy has also been rediscovered in Western countries, driven by the problem of growing antibiotic resistance.

Phages are viruses that infect and replicate in bacteria. Like other viruses, they are classified into families based on morphology and type of genetic material (DNA or RNA). Most of them can be described as 'tailed phages', which have double stranded DNA. Another classification is based on their infectious cycle, which is lytic or lysogenic. Both cycles start with attachment of the phage to receptors on the bacterium's cell surface, which determines the phage's specificity, followed by the injection of the viral DNA into the bacterium. At this point the lytic cycle and the lysogenic cycle differentiate.

To produce Lytic phages the bacterial transcription and translation machinery starts to express the early virus genes that are needed to copy the viral DNA and proteins. New bacteriophages are then assembled and finally expression of the phages' late genes lead to production of lysins and other enzymes, that result in lysis of the bacterial cell and thus the exit of progeny virions [45,47]. Lysogenic phages (also called temperate phages) on the other hand integrate their genome into the bacterial chromosome and remain dormant as a prophage. This process sometimes allows the transfer of virulence factors [43,49,50] such as toxins [40,51] making the bacterium that is infected with a virus more pathogenic. Therefore, these phages are unsuitable for phage therapy. The dormant prophage is copied when de bacterium divides, or is replicated when it enters the lytic cycle, triggered by circumstances that threaten the host bacterium, such as DNA damage or temperature [47].

The use of bacteriophages for treatment is determined by the infectious cycle: most commonly, bacteria are killed by bacteriolysin during the lytic process, while replication reinforces the virus' presence [40]. This way the bacteriophages are effective as long as target bacteria are present in sufficient amounts [43]. Alternatively, non-lytic bacteria that are genetically modified to contain a restriction enzyme instead of a bacteriolysin gene have also shown to clear infections *in vitro* and in mice by digesting the bacterial nucleic acids [48]. An added advantage of the use of these modified phages is that only a low amount of endotoxin is released compared to lytic phage therapies, resulting in higher survival rates due to a smaller inflammatory response [48].

In all cases, phages are very selective in targeting bacteria: often they are specific at the strain level [40,45,46,49]. This gives the use of phages for therapy two advantages: it does not affect mammalian cells [40,45] and it does not create dysbiosis because the commensal bacteria are not targeted either [43,45,51,52]. Taken together this will diminish the likelihood of adverse effects. However, there is also a significant drawback associated to this specificity: in order to treat an infection with phage therapy, the pathogen must be positively identified or there should be a high suspicion of its presence [43,45]. This means that it is important to isolate and culture the pathogen for identification, which is time consuming. At the same time, phage therapy is most effective when started shortly after infection [43].

Because the mechanism of bacteriophages is completely different from conventional antibiotics, they are still effective against multi-antibiotic-resistant bacteria [54], such as MRSA [53]. Unfortunately, this does not mean

that bacteria cannot become resistant to the phages as well. Several resistance mechanisms have been observed [45,50], such as the adaption of the bacterial cell surface receptors required for phage attachment [45,49,53,54]. To prevent resistance some researchers suggest to use phage cocktails, which contain multiple phages that target the same strain, making it less likely that the development of phage resistance may occur [43,49,54]. Others pose that this problem is less severe with bacteriophages than with conventional antibiotics, because bacteriophages co-evolve with the bacterial targets [40,48,54]. A third option is given by the abundance of different phages in the environment: isolation of new active phages would also circumvent existing resistance [45,47]. Finally, isolated lysins could be used instead of whole bacteriophages [49,50]. However, this would be without the benefit of self-propagation of the therapy. Because lysins are often as specific in their targets as bacteriophages, all the benefits of a narrow spectrum still exist [51,53].

A last disadvantage are the complicated pharmacokinetics. Bacteria have to be accessible for the bacteriophages, and the phages should reach their target. Topical administration has been shown effective, but translocation complicates matters and is an important consideration in phage selection. Still, there is evidence that intramuscular administration has succeeded to deliver high bacteriophage levels in the blood, which is promising for successful treatment of systemic infection [52]. Another problem after administration could be neutralisation of the phages by the hosts' immune system [45,51]. On the other hand, building an immune response would require multiple administrations, and often phages only have to be delivered once for an effective treatment [51].

Applications

Even though phage therapy has been used in Eastern Europe since the 1920s, the pharmacokinetics, dosing and adverse effects have not been described. Therefore, there are no FDA or EMA approved phage therapies yet. At the same time, some phage cocktails have been approved by the FDA for use as food decontaminants, such as the cocktails against *Listeria* and *E. coli* O157:H7 [47]. Since the interest in phage therapy has grown, a few clinical studies have also been performed. A safety trial for a cocktail of phages against *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* showed no adverse effects, however it also did not increase the rate of healing [56]. In another study (double-blind and placebo controlled) a bacteriophage cocktail significantly reduced VAS (visual analogue scale) scores by 50% and bacterial counts by 80% for *P. aeroginosa*-related otitis in all patients in the treated group, and cleared the infection in two out of 12 patients [55]. Chan *et al.* created a list of recent publications reporting on phage therapy targeting *P. aeroginosa*, *E. coli*, *Salmonella enterica* subsp. *typhimurium* and others in a variety of entities (including food, animals and humans) [46]. Most studies show a reduction of colonisation (between 10 and 10^5 fold) or increased survival rates while others are reported the use of bacteriophages as not effective.

Bacteriophages have also been shown to decrease MRSA infection in mice, and when used in a hand wash to decrease the number of *Staphilococci* on the human skin [53]. They have also been used against *E. coli* in a number of food animals [50]. Possibilities in aquaculture are demonstrated by the use of phages against *Lactococcus garvieae* and *Pseudomonas plecoglossicida* in fish [53] and decreased mortality in shrimp due to vibriosis that was higher than treatment with conventional antibiotics [49].

Future outlook

Although phage therapy was invented in the early 20th century, descriptions of its efficacy were semi-anecdotal, no double-blind placebo controlled studies were performed as required for approval as medicines in this day and age. Research into phage therapy in Western countries was restarted in the 1980s; however, more is needed to reach the point of FDA and European Medicines Agency (EMA) approval of phage-based medicines. A complicating matter is the way bacteriophages behave after administration: they replicate and

spread during treatment. Although this is a benefit in treatment, it does require some consideration and perhaps special regulations.

Hagens *et al.* found an application for one specific non-replicating lysogenic phage [48]. This opens the door for using lysogenic phages (expanding the arsenal against bacteria) and the lack of replication would be a solution to the concerns surrounding *in vivo* replication. (and this would also be an area that would benefit from more research into new applications, whilst keeping an eye on the concerns that are rightfully associated to the use of lysogenic phages.

Nevertheless, phage properties, such as self-propagation of treatment; selective, narrow spectrum and the lack of observed side effects, make them promising candidates for the treatment of multi-drug resistant bacteria. The possibility of selecting phages for a tailor-made cocktail is interesting, as the focus on personalised medicine is growing and is likely to keep growing in the future.

Finally, as with conventional antibiotics resistance will remain a problem. To provide an effective treatment it is important to monitor developing resistance mechanisms and to keep interest in the isolation of new phages, either by finding them in the natural environment or by development and targeted selection in laboratories.

Summary and Conclusion

The availability of antibiotics since the 1930s has had a great positive impact on the possibilities of modern medicine and agriculture. Unfortunately, the wide-scaled use of these antimicrobials has led to increasingly resistant bacteria, making infections more and more difficult to treat. To avoid being thrown back in time in terms of treatment options for bacterial infections, it is important that new antibiotics and alternatives are studied and developed into new forms of treatment or prevention.

In this paper, I have tried to provide an overview of some promising alternatives: antimicrobial peptides, from animal or bacterial origin; probiotics; and bacteriophages. For each of these options I have tried to review some potential applications. Are they more suitable for therapy or preventative measures? Is their use feasible at all? Can they be used on their own or should they be used in combination with other therapeutics? What could we expect from this alternative in the future?

Antimicrobial peptides are small peptides produced from eukaryotic (host defence peptides(HDP)) or prokaryotic (bacteriocins) origin. Both have a bactericidal effect, although their modes of action and specific targets differ. HDP also have advantageous immunomodulatory properties. Because the mechanism of action on bacteria is different from conventional antibiotics, both are not affected by the existing resistance mechanisms. However, bacteria have been shown to develop new resistance mechanisms to these peptides as well. Depending on the peptide, its spectrum may be narrow or broad with all benefits and disadvantages associated to activity spectra. Toxicity remains a concern, as does *in vivo* stability.

There are some current applications of bacteriocins: gramicidin S is limited to topical treatment; nisin has been safely used as a food preservative since the 1980s and lacticin-3147 is used in products for the prevention of bovine mastitis. A last hurdle is proving an efficacy higher than the current standard, as is required for approval of new medication. Keeping the current applications and properties of these peptides in mind, future applications are most likely to be either as therapies, decontaminants or as preservatives. In addition, the immunomodulatory effect of HDPs and the pore formation caused by many HDPs and bacteriocins could prove valuable for treatment in combination with conventional antibiotics. However, development of resistance makes it unwise to use these as a prophylaxis.

Probiotics have been used throughout history: yogurt was known for its health benefits in ancient times. In the 19th century bacteria were found in yogurt, and by the 1950s the term 'probiotics' was introduced. The definition has changed over the years, accommodating increasing knowledge about these bacteria. It is important that they are non-pathogenic to the host, able to survive the digestive system and antagonise pathogenic bacteria. This antagonism is the result of i) competitive exclusion; ii) bacteriocin production; or iii) immunomodulation. There is also some evidence that they inhibit virulence factors of pathogens.

Although probiotics have been proven to increase survival rates of poultry infected with *Salmonella typhimurium* and *E. coli*, they are applied as disease prevention more often. They can prevent diarrhoea from *E. coli* in pigs, reduce food-borne pathogens *Salmonella* and *E.coli*, and reduce *Campylobacter jejuni* shedding in poultry. In humans probiotics may be helpful in preventing *Clostridium difficile* infections associated with antibiotic use and diminish antibiotics' side-effects. Finally, they may substitute antibiotic growth promotors. *Baccillus subtilis* supplementation has resulted in similar weight gain effects as traditional antibiotic growth promotors. Considering the enormous amounts of antibiotics used in agriculture for treatment, but also for disease prevention and growth promotion, there is a large opportunity for decreasing the amount of antibiotics through substitution by probiotics. Further progress can be made by research into *in situ* bacteriocin production by probiotics, combining the two alternatives described above.

The last alternative are bacteriophages. These viruses were discovered in the 1910s, and were experimentally used for therapy until conventional antibiotics were developed. Phage therapy was rediscovered in the 1980s, driven by the growing problem of antibiotic resistant bacteria. For therapy, use is mostly limited to lytic phages because lysogenic phages may introduce new toxins and are able to transfer virulence factors between bacteria. The advantages of phages are clear: they are very specific, which means host cells and commensal bacteria are spared. It does however mean that pathogens must be identified before treatment commences. Although phages are able to kill antibiotic resistant bacteria, resistance mechanisms against phages are known. However, phages and their target bacteria co-evolve, which means regular isolation of new phages could be a solution. Another way to decrease resistance problems is the use of phage cocktails. Finally, it is possible to use lysins isolated from phages instead of whole phages. Another challenge are the pharmacokinetics: bacteria must be accessible and neutralisation of phages by the host's immune system could become a problem. Still, neutralisation requires multiple administrations and phages often provide an adequate treatment after a single dose.

Some phage cocktails have been approved by the FDA and EMA for use as food decontaminants, but as of yet, therapeutical applications of bacteriophages have not. A few trials that have been performed did not show adverse effects, and research on phage therapy in ongoing. It is likely that phage therapy will be an option in the future, especially for easily accessible infections, for example on the skin. Bacteriophages have been used to combat *E. coli* in food animals and promising results have been observed in aquaculture, where they were successfully used against pathogens for fish and shrimp as well.

Overall, these alternatives seem to have the potential to substitute (part of) the antibiotics used in the various fields described in the introduction. In human medicine, probiotics can be used preventatively, especially after treatment with broad spectrum antimicrobials. There is evidence they may be of use therapeutically as well. For therapy both AMPs and bacteriophages show potential. AMPs have the advantage that they provide a stable quality. Generally speaking, they are also available in a wider range of specificity, which is advantageous when the exact pathogen is unknown. Bacteriophages on the other hand, are more specific and require a strain-specific identification of the pathogen. Another problem is that they replicate in the patient after administration and may evolve during treatment, which makes them less constant in terms of quality. Whether or not this is an acceptable risk should be studied carefully. Either way, it may be a problem in terms of medicine approval and regulations. Additionally, national health insurance policies will aim to use the most economical treatment methods that are available. That will also be a deciding factor in the future of these alternatives for antibiotics.

In agriculture, probiotics can be used for growth promotion and disease prevention. AMP and bacteriophages would be useful to treat existing infections. Which of these two becomes the standard will depend on economic or regulatory factors. I have not discussed information on using these alternatives on plants, however given the prevalence of bacteriophages in environments that contain bacteria, they are likely to be found and isolated. Since AMPs have broader spectra, one could imagine using these as well.

On the other hand, given that resistance mechanisms have been observed for both AMPs and bacteriophages, one could wonder if the use of these alternatives in agriculture is desirable. The prevalence of antibiotic treatment, both in agriculture and medicine, have led to the situation as it is today: resistant bacteria become more wide-spread and increasingly hard to treat, even with last resort medication. Finding new ways of treating these infections would be very valuable in human medicine, and one might want to reserve these measures for our own species. At the same time, ethical considerations force us to treat our food animals and pets humanely. Is it acceptable to let these animals suffer from infections that might be curable with modern methods? What do we do when a bacterial pest destroys the harvest of staple foods in a large area? These are the big ethical and political questions that will need answering as these alternatives

become available. The answers will depend on public opinion, but it is up to the experts to make sure the information is widely available and easily accessible in order to create the most balanced, informed debate possible.

Summary for Laypersons

Modern medicine received a large boost when antibiotics became available in the 1930s. Nowadays, antibiotics are not only used for prevention and treatment in human medicine, but also for prevention, treatment and growth promotion in animals and treatment of plant disease in food crops. This widespread use has resulted in a rise of resistant bacteria. MRSA has become a well known hospital infection, and in the last year we have had reports of ESBL and resistant *Klebsiella* outbreaks in hospitals and retirement homes. At the same time, it has been a long time since new antibiotics were brought to the market. The only way to keep our health care at its current standard, is to develop alternatives for the current antibiotics.

In this paper, three alternative strategies are discussed. Probiotics, the 'good' bacteria, are suitable for the prevention of disease in both animals and people. By taking up free space and producing compounds that are toxic, they make it harder for the 'bad' bacteria to settle and cause disease. On top of that they help the immune system to fight against the 'bad' bacteria as well. They are also a good alternative for antibiotics to increase the growth of food animals in the meat industry.

A second alternatives are peptides produced by animals, plants and bacteria that are toxic to bacteria. By isolating or synthetically producing them, we could develop new treatment methods against bacterial infections. Before these peptides can be used in the clinic, some problems have to be solved: 1) they are sometimes toxic to humans and animals as well, and 2) a solution needs to be found how to deliver the peptides in their active state at the infection. Finally, bacteria will become resistant to these peptides as well. This means the search for new antibiotics will not be over when these compounds are ready for common use.

The last option is using viruses that infect and kill bacteria only. These so-called bacteriophages are found wherever you can find bacteria. They are very specific, which means that there are phages which target only 'bad' bacteria, leaving the 'good' bacteria in our body unaffected. Although bacteria can become resistant to bacteriophages, there are many different phages available that replicate and evolve themselves. Therefore, new bacteriophages will be produced that can be used against 'bad' bacteria that cause disease.

Finally, we have to keep thinking about how our society deals with the problem of resistant bacteria. Do we want to make new treatments available for human use only, or do we allow veterinarians to use them in their practice as well? Is it humane to let animals suffer from disease if new medication might be able to relieve them? What do we do when bacteria threaten our crops or food animals? Would that warrant the use of medication otherwise reserved for humans? Should the United States ban the use of antibiotics for growth promotion, like the European Union has done? How can we stimulate research for the development of new antibiotics and alternatives? These are all questions that should be answered by the public, and the answers will lead to rules and regulations that can make or break our future in a world full of bacteria.

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