Bacteriophages for improvement of intestinal health in Pigs & Poultry

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Abstract

Since the ban on antibiotic use for growth promotion, bacterial infections of the intestines are an emerging problem in poultry and pig farming. In addition, there is increasing pressure to find better alternatives to antibiotics in the battle against bacterial infections. The use of bacteriophages or endolysins in animal feed might be such an alternative.

In pigs and poultry a relatively small group of bacteria exists, which form the major threat to intestinal health. Therapeutic phage/endolysin treatment of some of these intestinal pathogens; i.e. *Brachyspira* ssp, *Clostridium perfringens, Clostridium difficile* and *Escherichia coli* might be an option.

However, bacteriophages and endolysins are not equally applicable for all these infections. Various phages and/or endolysins have been isolated, characterised and tested *in vitro* and sometimes *in vivo*. They vary greatly in, specificity, virulence, lytic potential, sensitivity (for light, pH-value) per individual phage. Therefore, each phage has to be considered independently for its potential to survive residency in animal feed, and the oral application route and finally its efficacy to reduce or eliminate the specific pathogen. Furthermore, the distinctive properties of targeted bacteria affect the suitability of the therapy.

Overall, the limited research, and not the possibility, has hampered the use of phages for such therapeutical application. While bacteriophage and endolysin treatment will probably never fully replace antibiotics, they can be a good addition to and might be used in combination with them, to combat the increasing bacterial infections in pigs and poultry.

Contents

Introduction

In recent years research has successfully been conducted in supporting bacterial flora in the digestive tract of pigs and poultry through nutrition [1]. Intestinal infections are a serious threat to the welfare of these animals. There are two types of infection; the first is caused by specific pathogens such as *Brachyspira* ssp, *Clostridium* ssp and *Escherichia coli* [2]. The second is the non specific bacterial enteritis where outgrowth of certain bacteria causes a disruption in the bacterial flora, so-called disbacteriosis. Possible factors contributing to intestinal sensitivity for disbacteriosis include feed interruptions, dietary changes and subclinical coccidiosis [2]. Antibiotics can help prevent these types of infections.

Until recently, antibiotics were being used on a very large scale in livestock. In 2006 however, the European Union began to restrict its use by prohibiting application of antibiotics as growth promoters [3]. Moreover, Apart from the lateral damage to the bacterial flora in the gut caused by unspecific targeting, resistance to these antibiotics is an increasing problem. To put things in a broader perspective; in 2007 up to 35% of all *Staphylococcus aureus* infections in the UK were methicillin resistant, which is correlated with multiple drug resistance (http://www.rivm.nl/earss/database/). This clearly demonstrates the need for 'new' alternatives to antibiotics in livestock. The use of bacteriophages might be such an alternative.

In 1917, Felix d 'Herelle discovered viruses which could infect and kill bacteria, the so-called bacteriophages [4]. Though potentially promising as a therapy, they were pushed aside in the Western countries and the US when antibiotics were discovered in the 1940's. In the Soviet Union and Eastern European countries however bacteriophages have been extensively studied [5-7] and used for therapeutic purposes in humans (especially in the army) throughout the 20th century. They were applied as an alternative for, or in combination with, antibiotics and when used correctly, were proven to be most effective [8]. In addition, lytic phage enzymes, endolysins, have recently been isolated and effectively used as antimicrobials [9].

In the Western world phages came back into the limelight only during the last two decades. Now, bacteriophages are already successfully being used in food preservation [10, 11]. Research has pointed out they are a potent and safe way to control bacteria such as *Lysteria monocytogenes*, and to a lesser extent *Escherichia coli*, *Salmonella enterica,*and *Campylobacter jejuni* in the food-chain [12]. Likewise, several studies have been conducted applying phages or endolysins to target pathogens in animals, for the improvement of animal and human health. Though initial results were ambiguous, subsequent studies in animals proved promising [13-15], which we will elaborate on in 'Phage therapy for improvement of intestinal health'. **In this review we will summarize aspects of bacteriophage and endolysin therapy and discuss its potential for future application in animal feed for improvement of the health state of poultry and pigs.**

Bacteriophages

Bacteria are a very predominant life-form on this planet; however they are far outnumbered by phages. It has been estimated that more than 10^{31} phages exist. If strung together, the distance covered would be tantamount to 46 million times the distance to the nearest star (which lies over 4 light years away) [16]. Phages are not only the most abundant biological entities but probably also the most diverse [17].

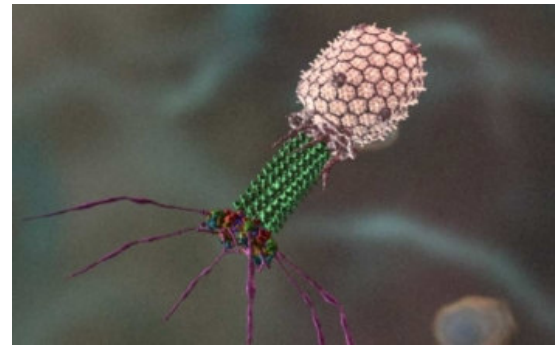

Figure 1. The bacteriophage T4 is preparing to infect its host cell. The structure of bacteriophage T4 is derived from three-dimensional cryo-electron microscopy reconstructions of the baseplate, tail sheath and head capsid, as well as from crystallographic analyses of various phage components. Adapted from *Purdue University and Seyet LLC*

Phages have in common that they only infect

bacteria. They inject their genetic information (either DNA or RNA) into susceptible bacteria where new phage particles can be produced. While the phenotype of the phages differs greatly, the make-up of each virion is relatively similar; it consists of a capsid with therein its genetic material [18-20]. The predominant group, 95%, of all known bacteriophages has a tail and dsDNA, as for example *E. coli* phage T4 depicted in **figure 1**. The remaining 5% is tailless with a genome that consists of either ssDNA, ssRNA or dsRNA [21].

The main effective differences between phages are their mode of entry, genome incorporation and release [19, 22].

Life cycle of a bacteriophage

Entry

The various types of phages can each infect a very narrow range of bacteria. With their distinctive receptor binding units they specifically bind to a host receptor. Phage adhesion sites vary greatly even between highly related strains. By recombination, new distinctive adhesion sites can be formed [23]. Phages for Gram-positive bacteria predominantly seem to adhere to specific teichoic acids embedded in peptidoglycan layer whereas phages for Gram-negative bacteria bind to a whole array of different membrane structures such as proteins, lipopolysaccharides or sugars [24-26]. Phage adsorption occurs in two steps. The first binding step is reversible; this might provide the phage with an opportunity to 'choose' whether infecting the bacterium is favourable. After the second binding step, the phage will insert its genome into the cell. This transfer is thought to be mostly a passive process [20]. The manner of insertion depends on the morphology of the phage. For instance, most tailed phages, such as *E. coli* phage T4, exhibit enzymatic activity at the tip of their tail, wherewith they breach the bacterial wall and are able to insert their genome through the tail into the host [27]. In comparison there are tailless phages whose envelop (which tailed phages do not possess) may for example fuse with the bacterial outer membrane and after the phage murein hydrolase has digested the peptidoglycan layer, they will pass the plasma membrane through internalisation [28].

Replication

When the genetic phage material resides in the cytoplasm of the bacterium 2 different major modes of action can take place [22]. Phage characteristics and bacterium physiology determine whether the lytic or lysogenic cycle is entered [22]. In **figure two** a complete overview of the lytic/lysogenic phage life cycle is given. There is also the chance of a phage causing a chronic infection, whereby virions are formed without causing cell lysis.

The lysogenic cycle

After infection the (temperate) phage can enter the lysogenic cycle [29]. When this cycle is entered, for instance due to low energy levels [30], the lytic genes are repressed and the phage coexists with host. This repression is regulated by regulatory proteins early in infection which bind to operators and thereby inhibit the transcription of lytic genes. The phage genetic material may either integrate into the host genome or exist as a plasmid in the cytoplasm. When the bacterium divides, the so-called prophage will be replicated with the host genome and also latently reside in the 'daughter' bacterium [19]. However, under influence of certain environmental changes, such as low energy levels, UV related DNA-damage, heat shock or other stress signals, a switch to the lytic cycle can occur [19].

If the prophage is integrated into the host genome and the phage enters the lytic cycle it is possible that parts of the host genome are excised with the phage and hereby of genetic host material will be transduced to a new bacterium. This is a positive development for the bacterial population as a whole, since this enables the spread of beneficial (e.g. virulence/resistance) genes. In, for example Salmonella, up to 5% of the genome consists of prophages, most of which include bacterial virulence factors [23]. Likewise, lysogeny renders an infected bacterium resistant to super-infection [31, 32], thereby protecting it from 'instantaneous' lethal lytic infection

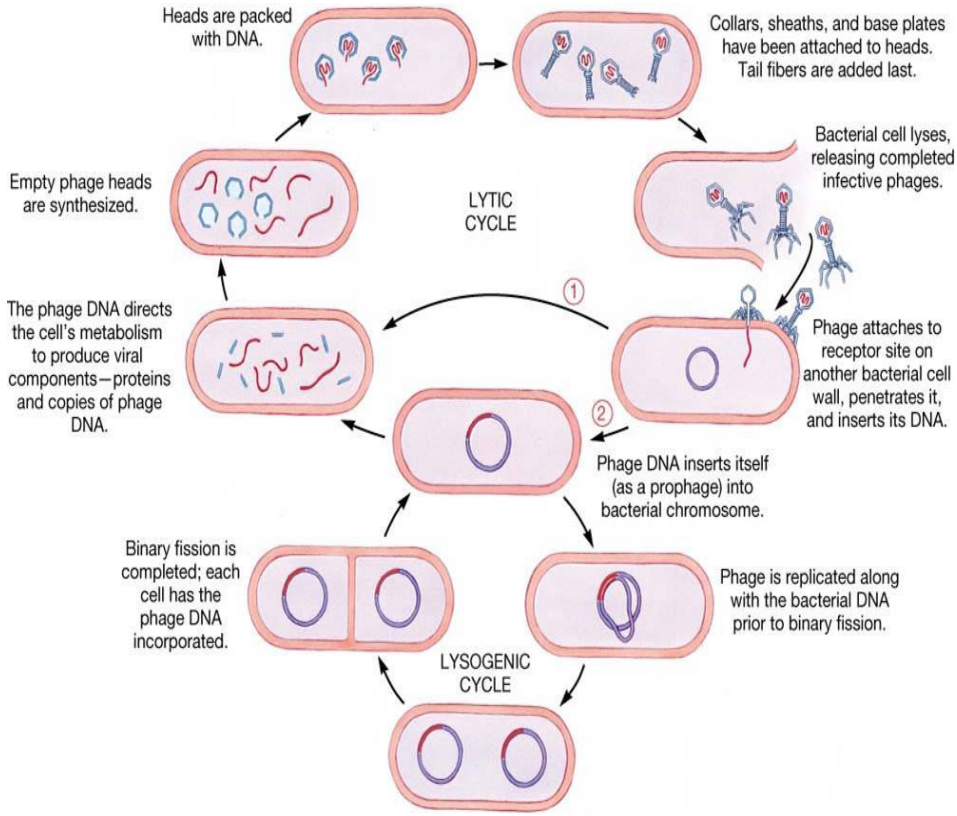

Figure 2. Phage species and bacterium physiology determine whether the lytic or lysogenic cycle is entered. Here a complete overview of their lifecycle is given. Adapted from the site of Prof. Theresa Fischer: http://faculty.ircc.edu/faculty/tfischer/micro%20resources.htm

The lytic cycle

If the lytic cycle is entered upon infection, instantaneous transcription of immediate early genes occurs. These can cause restructuring of bacterial metabolism, inactivation of host defences and protection of own DNA/RNA. Some of these genes when cloned give rise to products which are to some extent lethal to bacteria in their own right [33]. Subsequently, so-called middle genes are transcribed which give rise to products that produce new phage genomes. At that stage all major processes in the host have come to a halt exempting the cascades inherent to genome replication. Finally, late genes are synthesized which encode for the remaining virion particles [19].

A lytic cycle will take between 20-60 minutes, hereafter the cell lyses and the hundreds of newly formed virions move on to infect a new host. In tailed phages the lysis is commonly regulated by two proteins: an endolysin and a holin [21]. First the holins will form pores in the cell membrane which allows the endolysins to pass through and cleave the bacterial wall, causing lysis [19]. A second possible way of lysing the cell is through inhibition of peptidoglycan synthesis, thereby destabilising the cell wall [34].

Phage resistance mechanisms of bacteria

It is thought that bacteria and bacteriophages have evolved side by side over millions of years and in some cases have attained an almost symbiotic relationship, with the bacterium supplying the energy necessary to replicate and, as mentioned, the phages transferring virulence genes from one bacterium to another. On the other hand, lytic bacteriophages can be quite harmful and bacteria have developed numerous ways to evade infection. These can form potential barriers for phage therapies [9, 35].

Adhesion inhibition

To protect themselves against external threats some groups of bacteria can form a biofilm, as depicted in **figure 3**. This consists of a protective layer of so-called extracellular polymer substance to which the bacteria adhere. Though bacteriophage exposure is known to stimulate biofilm formation, this will only slow down but not prevent infection [36], since phages possess enzymes (in their tail) which can digest or depolymerise such barriers [37]. Likewise extracellular polysaccharides (EPS) and surface layer proteins potentially block or mask phage attachment sites. Surprisingly, they are also known to function as phage recognition sites. It seems the phages use this EPS to recognize more active cells, which provide a better environment for phage proliferation [38]. Phages are very competent at evading bacterial immune mechanisms and even appear to misuse these mechanisms for their own benefit.

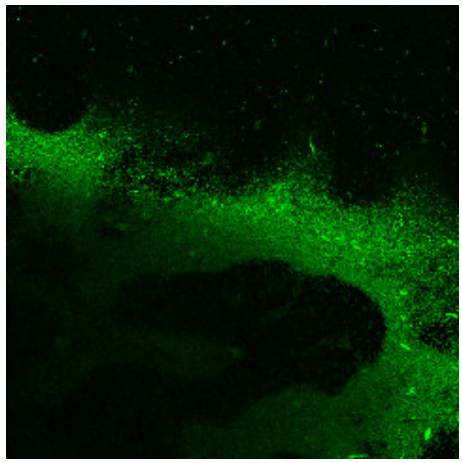

Figure 3. Image of a single slice through a biofilm from a confocal laser microscope.
Adapted from the Biofilm Research Group: http://www.edmstudio.com/biofilm/index.html

Bacteria further escape infection by lack of expression and mutations (alterations) of the phage docking sites [39]. While this might prove efficient for a specific phage, there are many different varieties of phages that can infect a specific bacterium. In addition, it is widely accepted that phages can easily adapt to these mutations, fore their mutation rate is significantly higher [18, 23, 40, 41]. In contrast, Lenski *et al.* reasoned phage adaptation to be less efficient, since this required a gain of function mutation whereas the bacterium required a loss of function to obtain resistance [42]. Nonetheless there are many phage subtypes within one population gainsaying the latter hypothesis [40].

DNA injection block

One step further down the line, DNA-injection blocking can also inhibit infection. The blocking has been associated with resistance to lysin in lactic acid bacteria [43], interference by cell membrane proteins and in alterations in membrane fluidity due to temperature [44].

Restriction modification

The nucleotides of the host (bacterial) DNA are methylated at the restriction sites of endonucleases, thus making them inaccessible. In contrast, the phage DNA will be recognized and demolished by bacterial endonucleases. Once more phages have adapted; by acquiring own methylases, inhibitors of endonucleases and eliminating/adapting restriction sites [35].

Abortive infections

Phage multiplication can also be averted by inhibition of the phage-related processes in the bacterium, such as transcription, translation, assembly of the phage etc. [44]. Ultimately, this will lead to auto-destruction of the bacterium, which ensures protection of the rest of the population.

Endolysins

Another, possibly better but less studied, option for new therapeutics are endolysins. These phage enzymes hydrolyse the bacterium wall and thereby facilitate phage release.

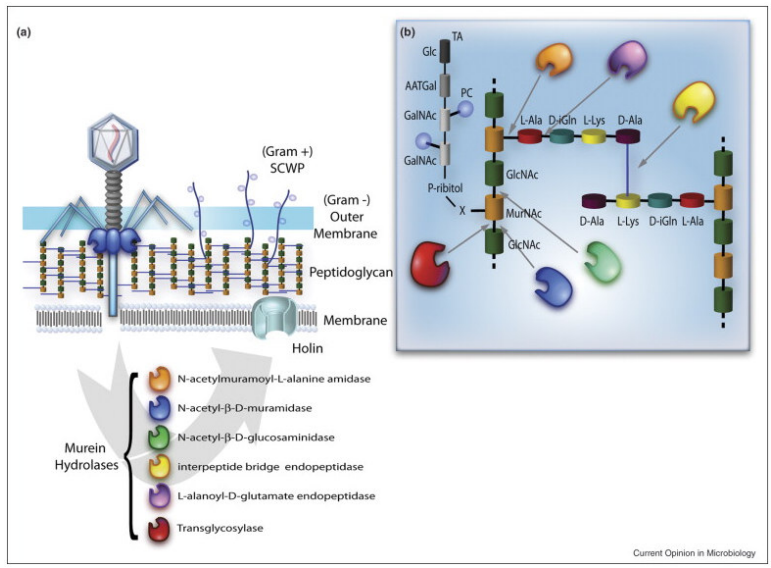

Figure 4. Bacterial cell wall structure and endolysins.

(a) After infection by bacteriophages, murein hydrolases encoded by phage genomes are produced during the late phase of the lytic cycle. Endolysins gain access to their substrate, the bacterial cell wall, when holin, a phage-encoded membrane protein, disrupts the membrane.
(b) Structure of peptidoglycan in *S. pneumoniae* with endolysin targets. Bonds cleaved by the different murein hydrolases are indicated by arrows. Adapted from Hermoso *et al.* [9]

Commonly, lysins are made up of two domains, the N-terminal catalytic domain which cleaves the cell wall and a distinctive C-terminal cell wall binding domain. Many different lysins are known to date, for instance lysozymes, glucosaminidases and endopeptidases. They can cleave various cell wall structures such as sugar moieties and peptides respectively, see **figure 4**. Some lysins such as T4 lysozyme, exert a dual-killing function. Apart from the peptidoglycan cleavage, they also contain a domain which promotes membrane disruption [45].

Most, but not all, lysins need 'holins' to transport them to the periplasmic space. After infection, endolysin accumulates in the cytoplasm of the bacterium; at a genetically specified time the small holin molecules form pores in the inner membrane through which the lysins can attack the cell wall and mediate release. A few exceptions have a N-terminal secretion signal and hitch-hike the host secretion system to cross the inner membrane [45].

Endolysins appear to be promising therapeutics as they specifically kill the Gram-positive bacterial species wherein they were produced, even when applied exogenously. Even though endolysins were first shown in 1957 to independently kill bacteria, it was not until the beginning of this decade that they were used therapeutically. In 2001, Nelson *et al.* showed that nine heavily infected mice had undetectable levels of group A *streptococci* two hours after oral administration of 500 lysin units of the streptococcal bacteriophage C [46]. The strong potential of various endolysins for therapeutic use was confirmed by studies in e.g. *Enterococcus faecalis, Enterococcus faecium,* methicillin resistant *Staphylococcus aureus* and *Bacillus anthraxis in vitro* and (though not all) *in vivo* [47-50]. It was even shown lysins could eliminate biofilms of *S. aureus* [51].

Endolysins are also more malleable than whole virion phages. By swapping catalytic or recognition domains of endolysins new recombinant forms can be designed. It has been shown with pneumococcal phage lysins that when active domains are interchanged, an active enzyme can be obtained [52]. Recently, scientists designed the functional chimeric endolysin P16-17 which targets and kills *S. aureus,* since there were troubles isolating the original lysin [53]. Such use offers opportunities for the future, where it might be possible to design novel lysins with distinctive properties and specificities. Also, in contrast with bacteriophages, no bacterial resistance towards lysins has been documented to date [45].

Bacteriophage/Endolysin therapy

Bacteriophages and endolysins can be applied therapeutically. By administering natural or genetically modified phages specific for pathogenic bacteria to a host, these bacteria can be counteracted or even completely destroyed. While phages are used in the former Soviet Union, bacteriophage therapy is not widely applied as treatment of animals and humans in the Western countries. This is predominantly due to a lack of research, negative public perception of phages and the very strict regulations imposed on their use [54]. For not only are they a potential alternative for antibiotics, they have shown to be most effective in their own right [5, 7, 14].

Antibiotics, as aforementioned, are rapidly becoming less effective in treatment of bacteria due to resistances. Bacteriophages or endolysins would be a very good replacement, since their recognition and destruction mechanisms are vastly different from antibiotics and thereby will circumvent any resistances the bacteria have obtained [55]. Also when applied in livestock, any bacterial resistance that might arise will not interfere with antibiotics treatment in humans. Moreover, bacteriophages are relatively easy to produce and therefore quite cheap.

While all very promising in theory, in practice it is not as clear-cut as it may seem; many variables play a role in determining the applicability of phages and endolysins as a cure. Not just their distinctive characteristics, but also the bacterial and host responses affect their therapeutic viability [9, 33, 55].

Phage/endolysin administration

In view of the highly specialised nature of the phages, it is important to determine the 'right' phage to use in for example animal feed or in preservation of food, since inactive phages are of no use.
Because of the broad scope of phages, they have very diverse survival conditions. There are a few common denominators however; all phages are relatively susceptible to ultra violet light, heat and a very high or low pH [20]. Most frequently phages are able to survive between pH 5 and 8, however *Enterobacter* and *E. coli* phages resistant to acids down to pH 3 have been characterised. Potential hazards which should also be taken into account, when added to animal feed, are: ascorbic acid, urea and alcohols.

Various factors determine the efficacy of phage therapy in animals; the persistence of the phage in the host, the number and concentration of the dosage(s). The dynamics of phage dispersal throughout the host and their ability to persist relies on the presence of the host bacterium.

A few challenges remain for oral administration of phages. To start with; the low pH in the stomach of animals might disable the phages. An often applied method to circumvent this problem is the de-acidification of the subjects' stomach by an antacid, which allows the phages to pass through this highly acidic organ without being deactivated. Another potential glitch is our incomplete understanding of the make-up of the gastrointestinal flora of animals which is very complex and varies per species. It is therefore not surprising that studies have shown phage therapy against intestinal bacteria does not always work. For instance, Chibani-Chennoufi *et al.* found viable bacteriophages were able to travel through the gut of mice but did not infect the corresponding indigenous *E. coli* present. Whilst they were perfectly capable of doing so *in vitro* [56]. All in all these issues should be taken into account but are in themselves not insurmountable.

In endolysins, though *in vivo* work to date is extremely promising, the delivery to the intestine might form a potential barrier. A solution being studied is endolysin expression by a non-pathogenic bacterium. Vaccines and cytokines have already been shown to be delivered to the intestine by *Lactococcus lactis*. For endolysins, Gaeng *et al.* demonstrated the potential of *Lactococcus* to express and secrete lytic enzymes, *in vitro* [57]. Strengthening this, active CD27L lysin for *C. difficile* was proven to be expressed in *L. lactis* [58].

Phages and endolysins exhibit several characteristics which might be exploited or must be circumvented to maintain the viability and heighten the efficacy of the phage therapy.

Specificity

Bacteriophages are very specific [22]. They will only target a particular strand of pathogens and not exhibit random killing of the intestinal commensals. However as a consequence, before phage application a thorough diagnosis must be determined to pinpoint the precise pathogen. Nevertheless the preservation of a healthy gut flora far outweighs this. Since they are very specific, more than one phage might be needed to eliminate different strains of the same pathogen. A possible solution is application of phage cocktails containing multiple phages.

Where bacteriophages infect a very narrow spectrum of bacteria, endolysins operate on a broader scale. In *C. difficile* it was shown that phages infected four of the tested strains in comparison to the 30 affected by the corresponding endolysin [58]. Though less specific, when tested no or hardly any activity towards intestinal commensals was found [46].

Virulence

To increase efficacy, phages and endolysins must be highly virulent and readily 'infect' and kill bacteria. In addition, another extremely important characteristic for phages is their ability to induce the lytic cycle. Not only because that ensures the cell will be demolished immediately, but also because lysogenic phages can transduce virulence factors thereby even potentially worsening the situation. If endolysins are used as a therapeutic there is no need to fear transduction of virulence genes between bacteria.

Self-limitation

Theoretically, relatively low doses of bacteriophages need to be used, since they will exponentially increase with every lifecycle. Another positive factor is that after the targeted bacteria are destroyed, there will be no host for the bacteriophages to propagate in. Thus, when the threat is defeated the phages will equally disappear [18]. Fiorentin *et al.* presented a beautiful example of this self-limitation, as depicted in **figure 5**. After inoculating three groups of birds with either Salmonella and SE PT4 bacteriophages, SE PT4 phages only or neither, they took 7 cloacal swabs wherein they checked for bacteriophage presence. For the birds which were administered phages but did not have bacteria to propagate in, none of the cloacal swabs taken after two days were positive for these Salmonella specific phages. This in contrast with the birds who did receive Salmonella, where the phages were found up to at least nine days later [59].

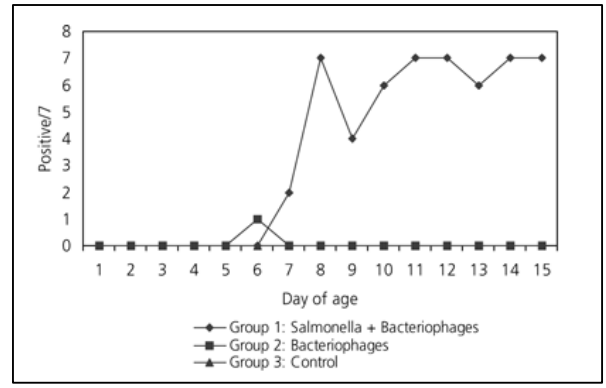

Figure 5. three groups of birds were inoculated with either Salmonella and SE PT4 bacteriophages, just SE PT4 phages or neither on day 4. Hereafter 7 cloacal swabs were taken every day, with on the y-axis the number of cloacal swabs positive for phage SE PT4. This picture was adapted from Fiorentin *et al.*[59]

A down-side of phage replication is that the bacterium must be active for the phage to multiply therein and eventually destroy it.

Endolysins possess no such replicative mechanism and will therefore experience neither the negative or positive effect.

Resistance induction

Phage therapy, while able to counter antibiotic-resistant bacteria, they induce resistance themselves, see the section 'resistance mechanisms bacteria'. A beneficial side effect of bacterial phage-resistance is that these bacteria might be less virulent *in vivo*, if the phage receptor is also a virulence factor itself. For instance, Smith *et al.* demonstrated samples of phage resistant *E. coli* obtained from the faeces of treated piglets, were less virulent than their non-resistant counterparts [14].

Moreover no resistance to endolysins has been discovered to date. Even when bacteria were challenged multiple consecutive times with sub-lethal doses of their corresponding endolysin, they did not develop resistance as was shown by Low *et al.* This finding has been connected to the targeting of highly conserved structures of the cell wall, since evolutionarily it is not very favourable for the phage to be trapped inside the bacterium.

Host immune response

Much research has been conducted towards usage of bacteriophages as a therapy in the former Soviet Union in animals and (predominantly) humans; hardly any allergic reactions or other negative side effects have been found [60]. On a cautionary note, while much research has been conducted not all has been done with the proper controls.

Not only bacteria have mechanisms evading phage infection. Also, the human or animal host can orchestrate an antibody response against them. Phages, when administered, are quickly taken up in the circulatory system and dispersed over the body. If they do not encounter bacteria wherein they can proliferate, they will be rapidly cleared, as first reported by Appelmans *et al.* in uninfected rabbits. However, when they encountered a suitable bacterium they have been found in the host (e.g. poultry) several days after infection [59, 61].

Both the host adaptive (formerly referred to as the reticulo endothelial system) and especially the innate immune system greatly influence the duration of phage persistence. The importance of the innate system for phage dynamics was demonstrated by Geier *et al.* in germ-free mice. After oral administration of phages their titre decreased nine-fold within the first hour and could only be detected in the kidney, spleen and liver. So, despite the absence of antibodies the phages were rapidly cleared [62].

Merril *et al.* found a way to circumvent the untimely elimination of the bacteriophages. They reasoned that if they isolated phages which survived serial passages through the host (in this case mouse) circulatory system, these phages would be less prone to be entrapped by the innate immune system. Herewith, they obtained two mutant variants of the *E. coli* λ phage which had 13,000 and 16,000 times more chance to evade detection than the wild-type strain [63]. On a cautionary note, these selection cycles where initiated by intra peritoneal injection. In a preliminary study when they tried to deliver the 2×10^9 plaque forming units (PFU) of W60 phages orally, none were recovered from the circulatory system.

Though the effect is not as significant as that of the innate immune system, antibodies seem to influence phage clearance too. Ochs *et al.* compared persistence of phages in severe immunodeficiency disease (SCID) and non-SCID individuals and clearly demonstrated increased persistence of phages in SCID individuals, who have, amongst other things, an impaired antibody response [20, 64, 65].

Yet, the antibody response may not present a major barrier. Orally en locally applied phages experience no or hardly any difficulties with antibodies. Anyhow, the effects of antibody neutralisation on phages are not clear-cut. Antibodies against specific phages have been found in 21% of the individuals before phage administration [20]. After treatment, antibodies were found in 54%. In total five patients did not recover with phage therapy and of these patients, two showed high antibody levels beforehand. However, the three other individuals showed significant health improvements and the overall accumulated antibody titres did not correlate with disease progression [66]. In animals, comparable amounts of phage specific antibodies have been found. Smith *et al.* demonstrated low concentrations of antibodies in only some

serum samples of calves and piglets. When these antibodies were orally administered combined with the corresponding phages this lead to lower concentrations of those phages in the alimentary tract [67]. In poultry, several studies reported no or little emergence of phage specific antibodies [68].

Since endolysins are relatively new with regard to therapeutical use, less research has been conducted towards their immunogenicity. Nonetheless, they are proteins and therefore likely to elicit an immune response. To investigate the possible adverse effects instigated by endolysins administration, various endolysins were injected into mice and rabbits. Not only did they find the resulting antibodies incapable of neutralizing the endolysins, also more importantly the animals themselves showed no signs of adverse side effects or anaphylaxis [69, 70].

Toxicity

Simultaneous destruction of large numbers of bacteria may result in the release of harmful amounts of bacterial endotoxins. As phages are of such interest, scientists have furiously been investigating ways to get around these set-backs. An example is the use of the modified non-replicating filamentous phage Pf3R, which efficiently destroyed wild-type *Pseudomonas aeruginosa in vitro*, while endotoxin release was greatly reduced compared to infection with the wild type phage. *In vivo*, the survival rate of the mice after a challenge with five times the minimal lethal dose was significantly higher when they underwent phage therapy with Pf3R rather than with the wild-type lytic phage [71].

Phage and Endolysin therapy for improvement of intestinal health

What is the potential of these therapies for improvement of the intestinal health state of poultry and pigs? To better consider this, one must look into the issues endangering the intestinal health of these animals. As aforementioned, intestinal disease is either caused by disbacteriosis or a specific pathogen.

Preventive treatment might not be an achievable line of action. Phages and endolysins infect a very distinct host-range, and therefore it is (almost) impossible to prevent outgrowth of an 'at random' bacterium. Especially since the intestinal microflora is very complex and due to the limited knowledge on its composition. Furthermore, phages are quickly filtered from the animal host after administration when no bacteria are available to replicate in and would therefore have to be administered daily, which seems unfeasible. This also leads to the next issue; the possible induction of neutralising antibodies. While seemingly not of enormous importance, it should be taken into account. Considering these arguments it may not be viable to use phage therapy to prevent intestinal infections in animals, perhaps exempting *E. coli*, which is habitually present in the intestine.

However, **therapeutic** treatment of the intestinal pathogens seems to be more attractive. In pigs and poultry there is a relatively small group of bacteria which form the major threat to intestinal health. Here we will discuss the research done to date for *Brachyspira* ssp, *C. perfringens, C. difficile, C. colinum, Salmonella* ssp, *Lawsonia intracellularis* and *E. coli* with regard to phage therapy and its potential for application in poultry and pigs.

Brachyspira ssp

Brachyspira ssp. are intestinal spirochetes which can cause diarrhoea in pigs and poultry. More specifically, the subspecies *Brachyspira hyodysenteriae* causes swine dysentery in pigs, which is a very severe haemolytic disease. Acute disease outbreaks have been described for meat pigs, with mortality levels up to 30% [72]. The other subspecies to be considered pathogenic in pigs is *Brachyspira pilosicoli*, however not only is it weakly haemolytic, it is also less abundant in the Netherlands and Belgium [73].
In poultry various *Brachyspira* ssp. can cause avian intestinal spirochetosis. This disease is characterized by diminished egg production and chronic diarrhoea. While the symptoms in poultry are less severe it still signifies a significant economic loss [74].

No published research has been done towards phage therapy for *Brachspira* ssp. Though, they have tried to isolate and characterize phages inherent to the *Brachyspira* ssp. VSH-1, a prophage like gene transfer inducing agent of *B. hyodysenterae* has been discovered [75]. This is a 'defective' phage which lacks capabilities to be 'infectious', that is when isolated and added to its corresponding bacteria it cannot form plaques. For, it lacks the ability to replicate independently. In retrospect, the early studies of for instance Humphrey *et al.* and Ritchie *et al.* who believed they had isolated lysogenic phages after mitomycin C induction, were mistaken [76-79]. The phages found for various *Brachyspira* ssp. were most likely also VHS-1 related prophage like particles. Not only did they equally fail to induce lysis when presented to bacteria, when characterized later they displayed great morphological similarities.

On the bright side, Matson *et al.* were able to sequence the VSH-1 associated DNA and thus discovered lysis genes [80, 81]. These encoded for holin and endolysin proteins. *In vitro*, the recombinant endolysin was able to hydrolyze peptidoglycan purified from *B. hyodysenteriae* [80]. It may be clear that while phage therapy seems not to be an option here, endolysins offer opportunities.

Clostridium difficile

C. difficile infection is an emerging problem in piglets [82]. When the microbiota of the gastrointestinal tract are unbalanced, this pathogen can cause *Clostridium difficile*-associated disease (CDAD) [83]. A common denominator for CDAD is diarrhoea; however infection can also be associated with a lack of symptoms or even constipation. Among the neonatal pigs examined for enteritis, 55% were affected by *C. difficile* [82]. When a herd is infected the morbidity is between 10 and 90% (generally 20 %) and the mortality of infected piglets up to 50% [84]. The clinical symptoms are caused by toxins A and B.

A very thorough study towards applicability of phage therapy in *C. difficile* infection was performed by Ramesh *et al.* [85]. They examined the ability of a bacteriophage, specific for *C. difficile,* to prevent infection in hamsters. Before administering the phages orally, the gastric acidity was neutralized by adding bicarbonate buffer, thereby enhancing phage viability. After being challenged with *C. difficile*, various groups of hamsters were administered either a single dose of 10^8 PFU or with multiple consecutive doses over various (short) time intervals. While all but one of the hamsters who received phage therapy survived, all the controls died [85].

However, the protection conferred through the phage therapy appeared short-term. Not only was there no bacteriophage recovery in the faeces two days post phage administration, indicating dispersal of bacteriophage from the hamster intestinal tract. Moreover, when the surviving hamsters were rechallenged with *C. difficile* two weeks after finishing bacteriophage treatment, they all died within 4 days. This can possibly be explained by the fact that the phage studied appeared to be a temperate phage instead of a virulent phage. Another important snag is that the phage used in this study was potentially able to transfer virulence genes.

Phage therapy for *C. difficile* is not a much researched area. So far only temperate phages have been characterized (due to interest for e.g. transfer of toxicity genes) [85-93]. The relative overabundance of temperate phages seems inherent to phages for (spore-forming) anaerobe bacteria. This is not at all surprising. Virulent phages need their bacterial host to be active in order for infection and multiplication to take place. Furthermore spores lack cell surface recognition sites for the phage. Therefore a lysogeny is a beneficial characteristic in phages for these bacteria, since prophages are not bound by the prevalence of active anaerobic bacteria in a frequently hostile environment [94].

Another limitation is the narrow host ranges all the studied *C. difficile* phages seem to have [86-88]. Of the phages isolated by Goh *et al.* phage C8 lysed 46% (26 out of 56 *C. difficile* isolates) followed the phages C6, C5 and C2 who destroyed a meagre 16-43%. This could prove problematic for application in therapy [91].

Luckily, a successful *in vitro* study has been done using endolysin of a Clostridium specific bacteriophage. Mayer *et al.* presented data indicating the endolysin of phage CD27 affected all 30 *C. difficile* strains tested, however not commensals of intestinal tract [58]. This lysin was active over a relatively broad pH, suggesting that it would preserve its functionality in an intestinal environment. In addition, Govind *et al.* recently characterised an endolysin of *C. difficile* phage CD119 [92], which offers possible opportunities, though its functionality has not been tested yet.

Clostridium perfringens

C. perfringens is the causative agent of necrotic enteritis (NE) in poultry. This pathogen commonly resides in the intestinal tract of bird species in very low amounts. However in case of intestinal disbalance it can proliferate and lead to onset of NE. The pathogenesis results from toxin production by the bacterium in the intestinal tract, which leads to tissue necrosis and often death. When left untreated, mortality within a flock can reach up to 40% [95].

Neonatal pigs can likewise be affected by this pathogen, where it can cause high mortality. Although the disease was commonly controlled through the use of antibiotics, the ban on their use as growth promoters has caused a re-emergence.

During the last century multiple experiments have been conducted characterising phages for *Clostridium* [96-112], yet many are far from practical for this purpose. Most are towards the applicability for typing of *C. perfringens* strains [97, 107-109, 111]. These *C. perfringens* strains may be defined by their susceptibility to a range of specific phages. Yet, studies have also described temperate phages arguably qualified for possible phage therapy [98, 100, 103, 106, 109, 110, 112].

Though no research towards entire virion phage therapy in *C. perfringens* has been conducted, Zimmer *et al.* have isolated the murein hydrolase of the *C. perfringens* associated phage phi3626 [113, 114]. This endolysin possesses the capacity to infect and lyse all 48 *C. perfringens* strains it was tested against, *in vitro*. Moreover other bacterial strains were generally not targeted [114]. Considering, that for *C. perfringens* predominantly lysogenic phages were identified, here endolysins seems to be the road to follow in therapy.

Clostridium colinum

C. colinum causes ulcerative enteritis or "quails disease". The symptoms vary between ulcerations of the intestinal mucosa and haemorrhagic enteritis [115]. This is a severe disease that occurs in quails with epidemic proportions [116]. In addition, ulcerative enteritis has also been found (sporadically) in other avian species such as young chickens [115, 116]. There, the mortality rate can rise up to 50% when left untreated [116].

One can be very short to the question: what is known for treatment of this disease with phages? Namely nothing. Since this disease has a relatively low prevalence in the more economically relevant avian species it has been underexposed and thus not much researched. However, if the other Clostridium species are considered, it would be logical to propose new research to focus on characterisation of their bacteriophages, whose endolysins might form a good basis for development of new therapies.

Escherichia coli

E. coli is commonly present in the intestine of most livestock and resides there without causing infections. However, it is considered to be the main causative agent in post-weaning diarrhoea in piglets, an often occurring disease. Though *E. coli* can equally cause enteritis in poultry, it is less abundant there.

E. coli is a well-known and researched pathogen with regards to bacteriophage therapy. A very illustrious early study performed, is that of Smith and Huggins in 1983 [14]. They surveyed the prophylactic and therapeutic properties of *E. coli* specific phages in enteritis of piglets, lambs and calves. Furthermore they examined their potency in treating respiratory *E. coli* infections in chickens. The main focus of their research is on calves, wherein they discovered that when treated prophylactically (after challenge and before onset of diarrhoea) with 1 $x10^{11}$ PFU/dose B44/1 and B44/2 (*E. coli* phages), diarrhoea was prevented in all animals. This differed significantly from the untreated controls, where up to 100% died. Nonetheless, phage treatment did not prevent illness when applied at onset of diarrhoea, but it did severely reduce the level of illness and subsequent mortality [14]. Interestingly, the study on phage treatment in **piglets** produced comparable results; 57% of the untreated animals died in comparison to the death of zero% of the animals that were treated at onset of diarrhoea [14]. While therapy did not eliminate all *E. coli* bacteria from the gut it seemed to reduce their numbers to 'manageable' amounts. In addition, some phage resistant *E. coli* strains were isolated from the calves and **piglets**, though when examined *in vivo*, they turned out to be less virulent than their wild-type counterparts [14].

Similar experiments have been performed using sheep and mice, with varying results. There seems to be a tendency of successful *in vitro* work; where they show the phages capacity to infect and kill the corresponding bacteria. Though, after examination of *in vivo* application of the same phages results can go either way, depending on the bacterial challenge, application pathway and concentration of phages administered. For instance, while Bach *et al.* showed no reduction in faecal shedding of target bacteria after oral administration of phages to lambs, due to insufficient phages reaching the intestine; Raya *et al.* observed significant reduction of intestinal *E. coli* after phage CEV1 was orally administrated [117].

A very elaborate study was performed recently *in vitro* and *in vivo* in mice. Chibani-Chennoufi *et al.* isolated Four T4-like *E. coli* phages with a broad host range from diarrhoeic children [56]. While *in vitro* the phages could kill the targeted bacteria *in vivo* they could not significantly reduce the resident *E. coli* intestinal flora, even though the phage was shown to pass the gastrointestinal tract unharmed and functionally unimpaired. Surprisingly, when the mouse was challenged with 'new' *E. coli* bacteria, phages could induce a marked reduction [56].

Likewise it was often customary to administer the phages and bacteria simultaneously [13, 63, 68, 85]. Thereby, as can be deduced from the previous example, obtaining biased results, since it would be more natural to administer the bacteria in advance, before application of the phages. Presumably, that would enable the bacteria to settle in the intestine and create their own 'niche', which renders them less sensitive to phage infection.

Apart from respiratory infection, *E. coli* can also cause 'infection' of the intestine in poultry. Recently, Xie *et al.* performed two studies to determine the toxicity and applicability of phage over antibiotics in poultry [118]. They orally administered 10^8 PFU/(g body wt) of Esc-A phage to 300 chickens for two weeks to determine the toxicity compared to a control group which received solely buffer. Not only did no animals die, the weight increase was similar; 66.7% compared to 67.5% respectively [118].

The findings on toxicity were recently confirmed in a slightly different setting. ControlVet, has already entered the market with an *E. coli* phage suspension, after toxicity tests. They inoculated two groups of 18 chickens with either crude phage lysate or sterile BPW solution and found no distinctive differences between the two groups (personal communication Marcel van Bergen).

To compare the efficacy of phage therapy with antibiotics Xie *et al.* formed three groups of 250 chicks each, raised in separate 'buildings'. Each chicken in group one was administered $1x10^5$ phages orally every day. In comparison with group two which received 1 mg/10 g body wt chloromycetin and group three which received neither [118]. The diarrhoea and mortality were recorded every week for three weeks. Not only was phage therapy six fold more efficient in preventing diarrhoea than the antibiotic in the first week, it also halved the amount of chicken deaths. In the second week this difference became even more profound with no infection in the phage treated group and, 12.4% and 25.8% in the antibiotic and control group respectively. By the third week hardly any infection of death could be found in either of the groups [118].

They performed an additional study whereby they examined the incidence of death caused by bursal disease. To their wonderment they found application of the *E. coli* phage Esc-A instigated at least a three fold reduction compared to the antibiotic and control groups. Supposedly, this was caused by the specificity of the phage therapy enabling the microflora to stay intact [118].

Recently, several lysogenic phages have been isolated in pigs and cattle, and characterized offering novel opportunities for broad-spectrum use of *E. coli* phages [119, 120].

Surprisingly, in contrast with for example *C. perfringens* and *C. difficile*, here research has been aimed at therapeutic phage application. While lysins of *E. coli* phages have been characterised [121-123], these have not been used as such. However, this is specifically due to the fact that Gram-negative strains are more difficult to treat with lysins.

Lawsonia intracellularis

L. intracellularis is the causative agent of intestinal hyperplasia. It has been known to target a wide range of animals. This intracellular enteropathogen invades epithelial cells and subsequently causes hyperplasia. This pathogen mainly targets pigs. There it can cause a wide range of diseases including porcine hemorrhagic enteropathy, porcine intestinal adenomatosis, regional ileitis and necrotic enteritis [124]. In for instance The Netherlands high numbers of animals are infected, however mostly chronically and thus without clinical features. Though, acute disease outbreaks are not uncommon, with morbidity up to 60% [125] and mortality even as high as 85.8% [126].

In poultry (with exception of ratites), *L. intracellularis* infection is very uncommon. In chickens, with uncharacterised enteric disease, no *L.intracellularis* was recovered from the faeces. Even when this pathogen was administered orally to one-day old chickens, they did not develop clinical symptoms [127].

As for *C. colinum*, nothing is known with regard to phage therapy for *L. intracellularis* either. The problem lies in the fact this bacterium is intracellular. This makes it extremely hard, if not impossible, for the phage to reach and infect it. Therefore bacteriophage and endolysin therapy might not be applicable against *L. intracellularis*.

Salmonella ssp

Salmonella enterica is the well-known causative agent of food related illness in humans. The main instigator of Salmonellosis in humans, *S*. Typhimurium, is a common bacterium in poultry and often considered harmless in mature birds [128]. Because of its role in human disease however, multiple studies have been conducted, investigating the potential of phage therapy for reduction of *S*. Typhimurium in chickens. *S*. Gallinarum and *S*. Pullorum are pathogenic in poultry. They can cause fowl typhoid and pullorum disease. Though mature birds are almost resistant, pullorum disease is extremely fatal to young birds. This is also the case for *S*. Typhimurium, Enteriditis and several other serotypes. Mortality in outbreaks may reach 90% if left untreated [128].

S. Typhimurium commonly resides in the intestine of pigs without causing disease. Though, clinical symptoms may occur in the post-weaning or (possibly) neonatal pig [129].

An early trial was conducted by Berchieri *et al.* who isolated several lytic phages for *S*. Typhimurium and explored their effect on the mortality rate on infected one day old chickens [68]. Only one phage (phage 2.2) caused a significant reduction in mortality. A one log decrease of the number of *S*. Typhimurium bacteria was observed in the small intestine and caeca 12 hours after challenge.

An interesting observation was done by Atterbury *et al.* They conducted trials wherein three broad spectrum phages were applied in antacid suspension against *S*. *enterica* serotypes Enteritidis, Hadar, and Typhimurium in chickens. The phages against Enteriditis and Typhimurium serotypes turned out to be highly effective with a decrease of 4.2 log colony forming units (CFU) and 2.19 log CFU within 24 hours respectively, though the phage against serotype Hadar was ineffective [130]. Higher concentrations of applied phages correlated with increased resistance, however these bacteria quickly reverted to wild-type *in vitro* and *in vivo* [130, 131].

The effectiveness of phage therapy towards Salmonella was confirmed by others [131, 132]. Fiorentin *et al.* measured a three and a half fold reduction of *S*. Enteritidis after phage infection in the caecal content and a significant weight gain in the chickens. Pathogen reduction remained at 25 days post treatment [131].

Several other research groups have similarly characterised lytic phages specific for various Salmonella ssp. *in vitro* and could be applied in therapy [133, 134]. Though, one must pay attention to effective isolation of phages and especially where they are obtained. Higgins *et al.* isolated seven lytic phages from broiler houses, however when testing several of these phages against Salmonella bacteria isolated from the same environment no replication could be found [135]. Therefore, these phages do not or only exert limited influence on Salmonella levels in commercial broiler houses.

Though phages have proven to elicit a reduction in Salmonella levels, the results for the long term are still somewhat ambiguous and depend greatly on for instance application method:
Borie *et al.* demonstrated a significant reduction of *S*. Enteritidis colonisation in the caecum of ten day-old chickens, ten days after challenge with specific phages through aerosol spray, but less so (if still significant) when the phages were applied through drinking water [136]. On the other hand Andretti *et al.* found a marked reduction in *S*. Enteritidis levels 24 but not 48 hours post challenge in the caecal tonsils of one day old chicks [137].

Another contrast was that Andretti *et al.* administered the phages post bacterial challenge while Borie *et al.* applied them prophylactically. Since Salmonella may proliferate intracellularly, the time of treatment has been shown to be of vital importance. Prophylactic or fast high titre phage treatment, after bacterial challenge, was markedly more effective than treatment delay [136, 137]. Probably early administration enables killing of the pathogens before they are internalised.

Most interestingly, Bielke *et al.* decided to solve the problem posed by the relative narrow host range of most bacteriophages [138]. After determining the host bacterium for 44 bacteriophages, they were tested for their proliferative capabilities in other genera. Two bacteriophages were especially promising, apart from their ability to proliferate in *Escherichia* or *Klebsiella,* they were also able to infect two and six Salmonella serotypes respectively [138].

This study suggests that there is a pool of bacteriophages which infect a broader host range. Selection of phages with 'dual' infectivity, for example a non-pathogenic bacterium and multiple *S. enterica* serotypes, may allow for the phage to be administrated in low amounts with the non-pathogenic bacterium to support its replication in the host. Thereby enabling longer survival of the phage in the host in absence of the pathogen.

Taken together, because of the short susceptibility of chickens to Salmonella associated disease and the tendency of *Salmonella* ssp to become unreachable for phages when internalised, here prophylactic treatment with phages could be efficient. In addition, the research conducted by Bielke *et al.* could contribute by allowing phages to linger, even in absence of the bacterium, ready to strike when they finally do encounter Salmonella.

Far less interest has been directed towards Salmonella phages in pigs. The danger of transmission of Salmonella bacteria to humans, whose most abundant transmission route in poultry is through eggs, is for obvious reasons less of interest with regard to pigs, though still present. O'Flynn characterised two lytic Salmonella phages of porcine origin, one thereof was resistant to low pH and thereby viable for oral administration in pigs [134]

Similarly, endolysins have been a very under exposed area, apart from characterisation *in vitro* [139-141], they have not been associated with therapy for *Salmonella* ssp.

Conclusion

The application of bacteriophages and endolysins in animal feed for improvement of animal health presents a great opportunity but also an enormous challenge.

Though bacteriophages have proven to be a viable option to treat various bacterial infections, they are not the most qualified to treat random disbacteriosis because of their narrow host range and their rapid dispersal from the animal in absence of a host bacterium.

Therapeutic phage or endolysin treatment has potential. In pigs and poultry there is a relatively small group of bacteria which form the major threat to intestinal health, namely *Brachyspira* ssp, *C. perfringen, C. difficile, C. colinum, Salmonella* ssp, *L. intracellularis* and *E. coli.*

Bacteriophages or endolysins are not equally applicable against all these infections. Various phages and/or endolysins have been isolated, characterised and tested *in vitro* and sometimes *in vivo*. They vary greatly in sensitivity (for light, pH-value), specificity, virulence, lytic potential etc. per individual phage. Therefore, each phage has to be considered independently for its potential to survive residency in animal feed, and the oral application route and finally its efficacy to reduce or eliminate the specific pathogen. Furthermore the distinctive properties of the targeted bacteria affect the suitability of the therapy.

E. coli phage therapy has already been extensively researched with the first products just on the market. Here, for both in pigs and poultry promising *in vivo* studies have been conducted, showing its potential with broad spectrum lytic *E. coli* phages, which are viable at pH 3 and thereby orally applicable.
This is equally true for Salmonella, with many successful studies conducted in poultry. In contrast with an earlier remark, it might be especially viable to use phage therapy prophylactically for *E. coli* and *Salmonella* ssp. These pathogens have ways to make themselves (partially) inaccessible to the phages in the intestine and it is therefore of vital importance to target them before they evade infection. In *E. coli* prophylactic use has already proven applicable, while the basic requirements for such therapy can be met for Salmonella [136, 138]. On the other hand, it is questionable whether such preventive treatment is feasible.

In comparison if one considers the two treatment options for *Clostridium* ssp. and *Brachyspira* ssp., the conclusion is that endolysin therapy in this stage has more potential No fully satisfactory phages have been characterised which could be used in therapy. 'Preliminary' endolysin research has been promising, with relatively broad but still specific targeting of the bacteria and no chance of transduction of virulence genes. In addition the possibility of engineering recombinant endolysins exists, circumventing any problems that might arise. On the other hand, endolysins therapy is relatively new and solutions for e.g. lysin delivery are still to be found.

Though, phage therapy is an option for most pathogens, *L. intracellularis* forms an exception. To date no phages have been characterised (possibly due to its inaccessibility, and due to lack of interest so far) and one can conclude at this stage that this pathogen does not lend itself to be countered by either phage or endolysin therapy.

Overall it is a lack of research, and not of possibility, that have hampered the use of phages for these therapeutical applications. While bacteriophage and endolysin treatment will probably never fully replace antibiotics, they are a good addition to and might be used in combination with them, to combat the rising difficulties with bacterial infections in pigs and poultry.

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