Research programme for the development of alternatives to antibiotics in livestock farming

Conclusions and lessons learned
Research programme for the development of alternatives to antibiotics in livestock farming

Conclusions and lessons learned

Programme coordinator Immuno Valley
Foreword Immuno Valley

As the Managing Director of Immuno Valley, it is with great pleasure that I may present the final results of the Alternatives to Antibiotics (ALTANT) research programme. Immuno Valley created this programme in late 2008 at the request of – and in close cooperation with – the former Ministry of Agriculture, Nature and Food Quality, now the Ministry of Economic Affairs. The process that led to the full-fledged public-private partnership proved to be unique to the moment. The vital national interest in reducing the use of antibiotics in livestock farming resulted in governmental support in the development of alternative concepts and contracting it out to a public-private network such as Immuno Valley. This solidified the relationship with the companies that could turn any promising developments into actual products for the market. This formula was very successful and made the Immuno Valley ALTANT programme an example of an effective public-private partnership. This booklet describes the ALTANT programme, focussing on the second phase in which two promising projects were given the opportunity to further develop their ‘proof of potential’ work from the first phase into a product profile for the veterinary sector.

Both projects, Animal-Specific Immunomodulatory Antimicrobials (ASIA) and Evasion molecules in staphylococcal bovine mastitis VACCines (EVAC), investigated two distinct approaches as alternatives to antibiotics. Whereas ASIA focused on host defence peptides as anti-bacterial and immunomodulatory therapeutics, EVAC aimed at developing a vaccine targeting the evasion molecules of the bacterium instead of the bacterium itself. ALTANT phase II resulted in an impressive increase in scientific knowledge on molecular mechanisms underlying pathogen evasion strategies and host immune defence mechanisms along with novel insights how to employ this in fighting antibiotic resistance development. Although no actual products are in the clinical development phase yet, I am confident that the knowledge and expertise that was generated within the ALTANT programme will world-wide contribute to the research and development of promising alternatives for application in animal and human health.

I would therefore also like to express my congratulations and acknowledgements to the partners involved. The ASIA team from Utrecht University and Zoetis, as well as the EVAC team from University Medical
Center Utrecht, Utrecht University and MSD Animal Health, who both succeeded in achieving great progress in their understanding of the alternative strategies to antibiotics they aimed for.

Of course, the ALTANT programme would not have been possible without the support of the Ministry of Economic Affairs and the constructive monitoring and advise of the ALTANT Advisory Board, representing governmental and professional stakeholders dedicated to animal and human health. In particular, I would like to thank the chairmen of the ALTANT advisory board, Dr. ir. Albert Meijering, Dr. Jan Nijsten and Ing. Tonnie Greutink for the pleasant cooperation that Immuno Valley experienced in its role as programme secretary and coordinator.

Lastly, I would like to thank former Immuno Valley Managing Director, Dr. Arno Vermeulen and all members of the Immuno Valley team, who over the past years did an excellent job in program management, organizing ALTANT conferences and other communication and dissemination activities as well as their continuous dedication in writing and compiling ALTANT reports, including this very beautiful booklet.

I hope you will all enjoy reading this booklet and, of course, will benefit from the results achieved and lessons learned in this ALTANT programme!

Dr. Liana Steeghs  
Managing Director Immuno Valley
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foreword</td>
<td>4</td>
</tr>
<tr>
<td>1. The problem of antibiotic resistance</td>
<td>8</td>
</tr>
<tr>
<td>2. Working towards a solution: Introducing ALTANT</td>
<td>11</td>
</tr>
<tr>
<td>3. ALTANT phase II projects</td>
<td>13</td>
</tr>
<tr>
<td>3.1. ASIA</td>
<td>13</td>
</tr>
<tr>
<td>3.2. EVAC</td>
<td>21</td>
</tr>
<tr>
<td>4. ALTANT organisation structure</td>
<td>28</td>
</tr>
<tr>
<td>4.1. Programme management by Immuno Valley</td>
<td>28</td>
</tr>
<tr>
<td>4.2. Steering Committee</td>
<td>29</td>
</tr>
<tr>
<td>4.3. Advisory Board</td>
<td>30</td>
</tr>
<tr>
<td>4.4. ALTANT communication and dissemination</td>
<td>31</td>
</tr>
<tr>
<td>5. Conclusion: lessons learned and future prospects</td>
<td>33</td>
</tr>
<tr>
<td>6. References</td>
<td>37</td>
</tr>
</tbody>
</table>

Appendix I Scientific publications and patents                             | 39   |
Appendix II ALTANT in the media                                            | 42   |
Since their discovery in the 20th century, antibiotics have been the go-to treatment for bacterial infections in humans and animals. Because antibiotics were so effective, it led to widespread use. Unfortunately, this effectiveness is threatened by the fact that bacteria are well equipped to adapt to a new environment and will become resistant to antibiotics. The intrinsic complexity of the bacteria’s genome and its attendant flexibility make it possible for bacteria to avoid the selective pressure of antibiotics and even to develop variants that appear to thrive better in the presence of these medicines than in their absence.

Antibiotic resistance is a problem that threatens humans and animals around the world. It reduces the number of effective medicines for treating bacterial infections and poses the threat that infections may not be treatable at all in the future. In the EU 25,000 people die annually from an infection with resistant bacteria. This is accompanied by 1.5 billion euros in healthcare costs. Bacterial infections have also detrimental effects on animal welfare and come with huge costs for the veterinary sector. In livestock farming, antibiotic resistance development is a large problem. For example, in the Netherlands, 50% of Escherichia coli bacteria found in broilers are resistant to four or more classes of antibiotics. Unfortunately, the livestock sector does not have enough alternative strategies to deal with several of the more persistent bacterial infections. For instance, antibiotics are currently the only method for fighting Streptococcus suis or Staphylococcus aureus, bacteria causing meningitis in pigs and mastitis in cows, respectively. If these bacteria become resistant to all available antibiotics, this has serious consequences.

Persisting infections in the livestock sector are also a potential threat for humans, since there are resistant bacteria that can be transmitted from animals to humans. Whether or not antibiotic resistance in humans is a result of the use of antibiotics in animals is under debate. In general, micro-organisms are specialised in their metabolism and occupy very
specific niches in refined ecosystems. However, these niches are not always limited by the differences between species, such as between humans and other animals. These micro-organisms also possess a seemingly limitless ability to adapt to changing conditions. In today’s society, where humans and animals are part of a single ecosystem, even once specialised micro-organisms have been able to cross the barriers between species. Some examples include viruses such as HIV, SARS, and Influenza, but also bacteria such as Escherichia coli, Salmonella and Campylobacter. They have all adapted rapidly to changing environments. At the moment, a majority of all infections that pose a threat to humans have animal origins (zoonoses).

Overall, the urgency of the antibiotic resistance problem is clear, but the solution is not so easily defined. Developing new antibiotics could be the way forward. However, this might only be a temporarily solution, since bacteria could arise that acquire resistance for the new antibiotics as well. Moreover, at the moment there are not many new antibiotics in the development pipeline of the pharmaceutical industry and those that are being developed will be saved as a last resort for human healthcare.

Another solution is reducing the use of antibiotics. By using less antibiotics, the chance of resistance development is lowered. Unfortunately, the EU prohibition on the use of antibiotics as an antimicrobial growth stimulator in animal feed did not lead to a reduction in the use of antibiotics in livestock farming. On the contrary; between 1997 and 2007 the veterinary use of antibiotics in Dutch livestock farming increased by 83%\textsuperscript{5,6}. Therefore in 2007, former Minister Verburg of former Ministry of Agriculture, Nature and Food Quality (now: Ministry of Economic Affairs) presented a plan to reduce antibiotic use in the veterinary sector\textsuperscript{7}. The aim of this plan was to halve the usage of antibiotics by 2013 in order to lower the threat of development of antibiotic resistance. The plan consisted of measures such as creating awareness, prohibiting preventive administration of antibiotics, improved management of animals and better hygiene, which should decrease the number and spread of infections and antibiotics used. Indeed, between 2009 and 2014, the usage of antibiotics in agriculture decreased by 58\%.\textsuperscript{8} After this successful decline in antibiotic usage, a new goal was
set to further reduce the usage to 70%. However, this new target seems out of reach today since reducing antibiotic use in livestock farming by current measures is reaching its limit. After all, animals are still getting infected and will still need treatment. This creates a dilemma in which usage of antibiotics has the risk of resistance development, while infected animals still demand treatment. A solution for this dilemma is the development of new treatments for bacterial infections: alternatives to antibiotics.

The development of alternatives to antibiotics as a solution to the antibiotic resistance problem was already foreseen in 2008. It was recognized that more research was necessary in order to gain more insight into the development of resistance, the transmission of resistance, resistant bacteria and the development of alternatives for antibiotics. It was envisaged that alternatives to antibiotics could be developed as new antibacterial measures or as prevention measures, such as vaccines. The newly developed alternatives should fight bacterial infections, ideally without the risk of inducing resistance, and replacing the current use of antibiotics. Developing these alternatives is a complex process needing extensive research and development, making it a risky and costly undertaking. That’s the reason that the Ministry of Economic Affairs initiated the ‘Alternatives to Antibiotics (ALTANT) in livestock farming’ programme in 2008.
The mission of the ALTANT programme was to develop promising alternatives to the use of antibiotics and make them available for applications in veterinary healthcare. The ALTANT programme was set-up as a public-private collaboration in which knowledge institutes and companies work together, financially supported by the government. The collaboration should consist of partners specialized in veterinary and human health, since antibiotic resistance is a problem in both fields. ALTANT was therefore organized as a ‘One Health’ research and development programme. The One Health principle is based on the fact that human and veterinary medicine are inseparably linked to one another. By bundling knowledge and expertise from both disciplines, innovative solutions for dealing with current and new infectious diseases can be developed. Moreover, since similar diseases exist in animals and humans, similar solutions might be explored be it under the condition that there is no threat of developing cross-species resistance to these innovations. Also, when innovation leads to less bacterial infections in animals this might have a beneficial effect on human health. With this idea in mind, the Ministry of Economic Affairs asked Immuno Valley in 2008 to initiate a bundling of expertise in several project teams having a distinct innovative idea to research and develop an alternative to antibiotics. Due to the expertise possessed by the public and private partners within the Immuno Valley network, Immuno Valley was considered the most suitable organisation to realise the ALTANT programme’s ambitious goals (see also the ‘ALTANT programme management’ paragraph).

ALTANT PHASE I

In a call for proposals, Immuno Valley asked experts to form project teams of at least three partners and to submit a proposal aimed at realising a ‘proof of potential’ for an alternative to antibiotics. In total, 14 proposals were submitted and after extensive review by an independent evaluation committee, four projects were selected for ALTANT phase I (2009–2010). Each of the four projects represented a different line of development: Animal-Specific Immunomodulatory Antimicrobials (ASIA), Evasion
molecules in staphylococcal bovine mastitis VACcines (EVAC), MODulation with Immune-stimulating PHYtochemicals (MODIPHY) and REduction of *Streptococcus suis* infection by Use of Phages and LYSins (RESUPLYS). During phase I, the project teams got the chance to show ‘proof of potential’ of their alternative to the Steering and Evaluation committees, composed of technical, commercial and scientific experts. This phase was completely publicly funded by the Ministry of Economic Affairs. At the end of phase I, the results of the four projects were presented to industry and the evaluation committee formed by the Ministry of Economic Affairs. The projects were evaluated on their scientific progress, future industrial commitment and likelihood of valorisation. The evaluation committee unanimously concluded that the ASIA and EVAC projects offered the highest potential of realising an effective alternative for the use of antibiotics in livestock farming. Immuno Valley was asked to organise these efforts over the next few years in a public-private partnership, in cooperation with knowledge institutes and industrial partners, supported in part by a second subsidy from the Ministry. The other two ALTANT projects, MODIPHY and RESUPLYS, although not selected to continue into ALTANT phase II, also had interest from industry and continued in public-private partnerships, independently of the ALTANT programme.

**ALTANT PHASE II**

The aim of ALTANT phase II was to research and develop the alternatives to antibiotics from the ASIA and EVAC projects into a product profile ready for development. After agreeing to the scientific ‘proof of principle’ in phase I, the veterinary pharmaceutical industry committed to actively participate in phase II of the ALTANT programme. As such, ALTANT phase II became a collaborative effort of Utrecht University’s Faculty of Veterinary Medicine, the University Medical Center Utrecht, Zoetis (former Pfizer Animal Health) and MSD Animal Health, coordinated by Immuno Valley and financed in part (33%) by the Ministry of Economic Affairs. The total project budget amounted to approximately 18 million Euros, of which half was provided by the industry partners. Results of the ALTANT phase II projects are described in the following chapter.
ALTANT phase II projects

ASIA (Animal-Specific Immunomodulatory Antimicrobials)
3.1. ASIA (ANIMAL-SPECIFIC IMMUNOMODULATORY ANTIMICROBIALS)

GOAL AND BACKGROUND
The goal of the ASIA project was to develop novel products based on animal-specific host defence peptides (HDPs) for veterinary prophylactic and/or therapeutic use that could serve as alternatives to the currently used antibiotics for poultry and pigs. HDPs are small proteins that are produced by animals and humans, as an important factor of their own innate immune system. HDPs have a dual working mechanism: they can directly kill microorganisms but are also able to stimulate the host’s immune system and in this way indirectly disable microorganisms. This dual mechanism makes them unique antimicrobial agents and is probably the reason why resistance development against HDPs has not been reported, despite the fact that these peptides already exist in animals for millions of years.

EXPERTISE ASIA PROJECT TEAM
In ASIA, the group of Prof. Henk Haagsman (Faculty of Veterinary Medicine, Utrecht University (UU)) collaborated with multinational veterinary pharma company Zoetis (former Pfizer Animal Health). Prof. Henk Haagsman is an expert in molecular host defence. Zoetis is a world leader in veterinary medicine and vaccine development. The expertise in host defence peptides of Prof. Haagsman combined with Zoetis’ capability to translate fundamental knowledge into products made for a great collaboration.

ASIA RESEARCH LINES
Both chicken and porcine HDPs are studied in the ASIA project, but here we will focus on chicken Cathelicidin-2 peptides (CATH-2) and novel peptides based on CATH-2. CATH-2 is secreted by the immune cells of chickens and has antimicrobial and immunomodulatory functions. These characteristics have potential use in chickens since just after hatch, broilers have an undeveloped immune system, which makes them vulnerable for infections with bacteria like *Escherichia coli*. Treating chickens with CATH-2 before hatching, could potentially stimulate the immune system which makes the broilers less vulnerable against bacterial infections, thereby reducing the
need for using antibiotics. To unravel the application potential of CATH-2 as an alternative for antibiotics, the working mechanisms of CATH-2 was further elucidated by means of three research lines:

1. Investigation of the antimicrobial activity of CATH-2 peptides
2. Investigation of the immunomodulatory effect of CATH-2 peptides
3. In vivo challenge experiments to study the efficacy of CATH-2 peptides

RESEARCH LINE 1: Investigation of the antimicrobial activity of CATH-2 peptides

The objective of the first research line was to study the antibacterial effects of CATH-2 against important chicken pathogens, *Salmonella, Escherichia coli* and *Clostridium perfringens*. For this, *in vitro* experiments were performed in which different concentrations of CATH-2 were incubated together with bacteria. By using a unique combination of imaging techniques and binding assays, the antibacterial killing mechanisms of CATH-2 were studied.

RESULTS

The results showed that CATH-2 rapidly killed bacteria at very low concentrations (see figure 1a), making CATH-2 a very effective antimicrobial peptide\(^{10}\). It was shown that CATH-2 bound to the membrane of Gram-positive and Gram-negative bacteria, which resulted in morphological changes of the bacteria (e.g. membrane ruffling) and permeabilization of the bacterial membrane (see figure 1b)\(^{10}\). Permeabilization of the membrane of bacteria is detrimental for their viability, explaining the antibacterial effect of CATH-2\(^{10}\). In conclusion, the antibacterial activity of CATH-2 peptides makes them good candidates to be further developed into an alternative to antibiotics.
Figure 1. Antimicrobial activity of CATH-2.
A. *E.coli* was co-incubated with different CATH-2 concentrations for different timepoints and surviving bacteria were counted. Figure shows that *E.coli* was killed rapidly already at low concentrations of CATH-2 peptides.
B. CATH-2 induced morphological changes, permeabilization and ruffling of the membrane (shown by the protrusions), of *E.coli* determined by specialized microscopy techniques.

RESEARCH LINE 2: Investigation of the immunomodulatory effect of CATH-2 peptides
The second objective was to characterize the immunomodulatory effects of CATH-2. For this, a combination of *in vitro* experiments in chicken immune cells and *in vivo* experiments in chicken eggs and zebrafish embryos was chosen.

RESULTS
Using *in vitro* experiments in chicken immune cells, it was examined whether CATH-2 peptides can modulate the function and inflammatory response of these cells. Results showed that CATH-2 was able to induce and modulate the production of chemokines, important effector proteins of the immune system. Also the effect of CATH-2 peptides on toll-like receptor (TLR) activation was studied. TLRs are proteins that are expressed on cells of the immune system. They recognize specific molecules of microorganisms which lead to the activation of the immune system in order to clear the infection. It was shown that CATH-2 formed high affinity complexes with bacterial DNA and strongly enhanced activation of chicken immune cells (macrophages) via the TLR21 receptor, which specifically recognizes bacterial DNA. By stimulation of chicken immune cells with CATH-2 it was further demonstrated that the percentage of antigen presenting cells as well as the production of several factors involved in antigen uptake and presentation was increased (Kraaij et al, submitted).
Antigen presentation is a very important process of the immune system that is involved in ‘presenting’ foreign particles, such as bacteria, to specific cells of the immune system to provoke an effective immune response aimed at clearing the host from these foreign particles. Overall, the in vitro experiments showed convincing evidence that CATH-2 peptides have a wide-range of immunomodulatory capacity.

Following the observed immunomodulatory effects of CATH-2 in vitro a series of in vivo experiments was designed in which CATH-2 was applied in ovo and in zebrafish embryos. In ovo administration of CATH-2 peptides in the absence of bacterial infection showed no direct in vivo immunomodulatory effect and no adverse effect on intestinal development (Cuperus et al, submitted). However, in zebrafish embryos that were injected with several concentrations of CATH-2 and infected with Salmonella enteritidis, the CATH-2 peptides caused a delay in progression of the infection with Salmonella enteritidis (decreased bacterial load) and an increase in the number of phagocytic cells in the embryos. Phagocytic cells are important cells of the immune system that ingest and kill invading microorganisms, showing the immunomodulatory effects of CATH-2 and indicating that an additional (infective) factor is required for prolonged effects.13 Overall, these in vivo experiments suggested that CATH-2 peptides apparently exhibit minor immunomodulatory effects in the absence of an infectious agent, but can prime immune cells to be more responsive when challenged. This was studied further in research line 3.

RESEARCH LINE 3: In vivo challenge experiments to study the efficacy of CATH-2 peptides

To test the efficacy of CATH-2 in vivo, several chicken infection models were developed at Utrecht University and at Zoetis. In addition, drug safety profiles of CATH-2 were evaluated in ovo at Zoetis.
RESULTS
The in ovo safety study, in which 20-fold and 40-fold higher doses (compared to the in ovo therapeutic dose) of CATH-2 peptides were injected in eggs, indicated that using 40-fold higher doses of CATH-2 peptides resulted in negligible effects on egg hatchability by the peptides, showing that using CATH-2 peptides was safe. The first chicken in ovo challenge experiments, in which CATH-2 peptides were administered in ovo 3 days before egg hatch after which birds were infected with chicken pathogens Salmonella enteritidis or Escherichia coli, showed that CATH-2 was able to partially protect broilers from S. enteritidis and E. coli infection resulting in reduced mortality (see figure 2)\textsuperscript{14}, a reduced number of birds with clinical symptoms and a reduced severity of disease\textsuperscript{14}. These in ovo experiments showed the first proof of concept of CATH-2 as an alternative to antibiotic treatment.

Figure 2. Reduced mortality after in ovo administration of CATH-2.
Figure clearly shows that in ovo administration of CATH-2 peptides prior to a challenge with E.coli resulted in a reduced mortality (triangle) compared to chickens that were not treated with CATH-2 peptides (bullet).
CONCLUSIONS AND FUTURE PLANS
The ASIA project unequivocally demonstrated antibacterial and immuno-modulatory potential of CATH-2. A protective effect against bacterial infection when administered in ovo was found. However, if CATH-2 would be used in daily practice, it must be compatible with the in ovo vaccines already in use. Therefore, more studies on the efficacy and formulation of CATH-2 need to be performed. Given the proven antibacterial and immunomodulatory activities of CATH-2, Prof. Haagsman foresees that the peptide will find its application in the future: “I foresee a bright future in which the peptides we studied will be used as either immune modulating drugs that prevent infection or induce the immune system to clear an existing infection or as a bactericidal drug. I will keep doing research with these peptides.” Chris Zook, Senior Principal Scientist in Mechanistic Biology, Global Therapeutics Research at Zoetis is also positive: “The development of novel antibacterials is difficult and requires many years of effort for success. Through our interaction with Prof. Haagsman and his team, we believe that we have learned a great deal on how to exploit antimicrobial peptide substrate in the future.”

In conclusion, even though there is no fully developed CATH-2-based product yet, the gained knowledge has innovation potential and can be used to develop HDP-based antimicrobial and immunomodulatory products in the future. In the coming years, Prof. Haagsman will continue unravelling the immunomodulatory capacity of HDPs combining in vitro experiments and in vivo studies. These experiments will aim for identification of biomarkers for efficacy of HDPs and hopefully lead to further elucidation of the mechanisms responsible for the protective effect. That information can then be used to design tailor-made HDPs for use as an alternative strategy to treat and/or prevent bacterial infections.
LESSONS LEARNED ON PUBLIC-PRIVATE PARTNERSHIPS

Prof. Haagsman learned that it is possible to have a productive collaboration with industry, when the academic partner uses its expertise and technology to do ground breaking research, and the company has the resources to translate the findings into large in vivo studies. The translation from fundamental research to the clinic was also the challenging part of this collaboration. The fact that Zoetis joined the research in an early phase made it possible to do fundamental and applied research at the same time. Haagsman: “The collaboration was very satisfying. Zoetis let us do what we do best, which is fundamental research. Zoetis understood that a better understanding of the mechanism of HDPs would make it easier to develop a product.” Chris Zook underlines the importance of public-private partnerships in the world of breakthrough medicines and vaccines. Zook: “Partnerships foster creativity, facilitate risk sharing and advance innovative sciences.” According to Chris Zook, the knowledge, skills and experience with HDPs of Prof. Haagsman complemented their research program and their collaboration is very valuable for exploration of non-traditional approaches to combat infectious diseases. Next to the exciting scientific and innovation impact that was achieved, the ASIA project enabled three young researchers to obtain their PhD. One of the former PhD students, Dr. Tryntsje Cuperus, commented on the partnership with Zoetis: “Although industry and academic partners speak different languages it is very important for a good collaboration to be open for this partnership. We got a lot of freedom from Zoetis to unravel the mechanism of CATH-2, while they complemented this research by performing in vivo experiments that can translate this knowledge into a product. The collaboration with Zoetis was really a bonus during my PhD, since I learned to look further than just my fundamental research.”
Evasion molecules in staphylococcal bovine mastitis VACcines
3.2. EVAC (EVASION MOLECULES IN STAPHYLOCOCCAL BOVINE MASTITIS VACCINES)

GOAL AND BACKGROUND
The goal of the EVAC project was to find a solution for mastitis, an infection of the mammary gland (udder), in cows. In dairy cattle, mastitis is a widespread disease, that affects animal welfare. Reduced milk production, milk quality and culling of chronically infected cows causes huge economical damage\textsuperscript{15,16}. Mastitis can be caused by multiple bacteria, such as \textit{Staphylococcus aureus} (\textit{S. aureus}), \textit{Streptococcus uberis} and \textit{Escherichia coli}\textsuperscript{17,18,19}. EVAC aims to develop a vaccine to prevent bovine mastitis, thereby limiting the use of antibiotics. The results of the EVAC project are also valuable for human healthcare since Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) infections can cause major problems in humans.

EXPERTISE EVAC PROJECT TEAM
Since \textit{S. aureus} causes problems in both animals and humans, the EVAC project is a typical ‘One Health’ project. This is also the reason why in EVAC scientists from the University Medical Center Utrecht (UMCU) and the Faculty of Veterinary Medicine of Utrecht University (UU) were working together. In EVAC, the group of Prof. Jos van Strijp (UMCU), who is an expert in the field of \textit{S. aureus} infection and immune evasion, collaborated with the group of Prof. Victor Rutten (Faculty of Veterinary Medicine, UU), who is an expert in the immune system of the cow. The EVAC partnership was further complemented with the industrial partner MSD Animal Health (MSD AH), a globally operating company in the field of veterinary vaccine development.

EVAC RESEARCH LINES
Development of a vaccine against \textit{S. aureus} able to induce an effective immune response against the bacterium has proven to be very complex since \textit{S. aureus} secretes molecules that help the bacterium to evade the host’s immune system. These evasion molecules are amongst others capable of preventing phagocytosis, a mechanism of immune cells to ingest and kill foreign particles such as pathogenic bacteria. By blocking phagocytosis, the
Evasion molecules help *S. aureus* to survive and multiply within the host, causing serious infections. EVAC took an innovative approach by designing a vaccine that is directed against the evasion molecules instead of the bacterium itself. If this approach is successful, the same principle could potentially be used against *S. aureus* in humans.

The EVAC project was divided in three research lines:
1. Identification of antigens and immunological targets
2. Vaccine optimization
3. Development of a challenge model to define a final vaccine composition

In contrast to in studies of human innate immunity, proper molecular tools for high speed and high throughput assays are lacking for the bovine species. The EVAC team has developed several tools and assays, such as antibodies, bacterial mutants, protein mutants, cellular isolation protocols, functional assays, and in vitro immunological assays, that facilitate EVAC research but can also be shared with other scientists in the field.

**RESEARCH LINE 1: Identification of antigens and immunological targets**

The objective of the first research line was to identify relevant antigens for inclusion in the vaccine by elucidating the host-pathogen interactions between mastitis-causing bacteria and the host’s immune system. In this effort, studies focussed on the ability of *S. aureus* to evade phagocytes and neutrophils, cells of the host’s immune system having an important function in the defence against bacterial infections.

**RESULTS**

Since *S. aureus* is the main causative agent (70%) of bovine mastitis priority was given to this pathogen. By using a biological screening technology, over 20 novel proteins secreted by *S. aureus* were identified that are potentially involved in evasion of the immune system. From these identified proteins, the extracellular fibrinogen binding (Efb) protein was shown to block phagocytosis of *S. aureus* by neutrophils *in vitro* as well
as in vivo (see figure 3)\textsuperscript{20}. Another evasion molecule, LukMF’, showed to kill neutrophils\textsuperscript{21,22}. These insights underline the importance of a vaccine that neutralizes the evasion molecules.

**Figure 3.** Efb inhibits phagocytosis of *S. aureus*.

Graph shows dose-dependent phagocytosis inhibition by Efb.

**RESEARCH LINE 2: Vaccine optimization**

In general, vaccines are applied via the parenteral (usually intramuscular) route. However, it is unknown whether this route is the most suitable to specifically increase intramammary immunity (immunity in the udder of the cow) at the site of bacterial infection. To gain more understanding on how to induce protective immune responsiveness, this research line focussed on defining effective routes of vaccine application and assessment of immune-stimulating compounds, so-called adjuvants.

**RESULTS**

In a vaccination study several vaccine application routes were studied, and their effect on the quantity of the antibody response as well as their neutralizing capacity on *S. aureus* was analysed. The experimental vaccine, used in these vaccination studies, consisted of the two *S. aureus* evasion molecules, Efb and LukM (one of the subunits of LukMF’), that were identified in the first research line (see above). It was shown that subcutaneous administration of the experimental vaccine at the mammary
gland resulted in a significant increase of antibody levels in serum and milk (see figure 4) as well as a significant increase in the neutralizing capacity of the antibodies, shown by increased phagocytosis of S. aureus. In other vaccination experiments, the effects of various adjuvants on antibody production and their neutralizing capacity was investigated. Adjuvants are substances that are added to a vaccine to improve the efficacy of the vaccine, by increasing the host’s immune response. Novel adjuvant combinations were designed and their efficacy was compared after vaccination in the udder or the neck of the cow. It was shown that subcutaneous administration of an alum-saponin-oil-based vaccine near the udder increased the antibody response. Taken together, these studies revealed that subcutaneous administration near the udder of a vaccine including a potent adjuvant, has potential as a one-shot strategy to increase intramammary antibody responses against bacterial infections.

![Graphs showing antibody levels in serum and milk](image)

**Figure 4. Antibody levels in serum and milk increases.**

Antibody levels in serum (A,B) and milk (C,D) following several immunization routes with Efb (A,C) and LukM (B, D) were measured. The graph clearly shows that subcutaneous administration (triangle) resulted in a significant increase of antibody levels in serum and milk.
**RESEARCH LINE 3: Development of a challenge model to define a final vaccine composition**

In this research line, the knowledge gained on antigens and immunological targets (research line 1) and vaccine optimisation (research line 2) were combined and the efficacy to prevent *S. aureus* infection of the preferential vaccine composition and application route were tested in a challenge model.

**RESULTS**

To evaluate the induced and the protective capacity of the immune response, a large set of *in vitro* immunological assays were developed, which were not yet available for bovine species but are essential in understanding the mode of action of the candidate vaccines and bovine immune responses. In the challenge model, one group of cows was vaccinated subcutaneously near the udder with a combination of antigens and toxins, including LukM, and another group of cows was not vaccinated. After vaccination, both groups of cows were injected with *S. aureus* in the udder. Efficacy of the vaccine was evaluated by monitoring the bacterial load in the milk and by measuring several parameters of the immune system, using the immunological assays mentioned above. Unfortunately, the final challenge trial did not yet result in significant protective effects.

**CONCLUSION AND FUTURE PLANS**

Although the development of a vaccine against bovine mastitis pathogens was not achieved, great progress was made in a number of areas that might find their way in future products or applications. Initially the goal was to develop a vaccine against bovine *S. aureus*, *S. uberis* and *E. coli* with *S. aureus* as the most important target. It was expected that findings from *S. aureus* research could be translated to *S. uberis*, however, this turned out not to be possible since the pathophysiology of *S. uberis* is quite different from *S. aureus*. This, together with the fact that studies in the cow are time consuming, made the EVAC team to take the decision to abandon *E. coli* and *S. uberis* and focus on *S. aureus*. The second phase of EVAC resulted in more insight into host-pathogen interactions and immune evasion by *S. aureus*. The knowledge obtained on induction of neutralizing antibodies in milk
and the elucidation of the molecular mechanism of two S. aureus evasion molecules is an important step towards the development of efficacious vaccines. Also, new animal models, in vitro assays and other molecular tools were developed that were not available for the bovine species before. These tools, together with the knowledge obtained on vaccination routes and adjuvants, will facilitate bovine mastitis research and vaccine development, even beyond the mastitis field, in the future.

LESSONS LEARNED ON PUBLIC-PRIVATE PARTNERSHIPS
Although no vaccine against S. aureus in cows has been developed, the EVAC project partners look back on EVAC in a positive way. Great scientific progress was made and this was allowed by the close collaboration and the joint effort of the project partners. Antigens, identified and characterized at the UMCU, were evaluated by MSD Animal Health using their facilities and technologies and were linked to knowledge on immunology and processes in the udder by the scientists at UU. Communication between the project partners was very efficient, which is very important when you depend on each other. During the project, changes had to be made in the initial planning. An important factor in the decision making of those changes was the Steering Committee. According to project leader Prof. Jos van Strijp, the Steering Committee was essential in EVAC and made the collaboration a success. Prof. Victor Rutten also looks back on the collaboration in a positive way: "We learned that a common goal and partners open for exchange of ideas and constructive criticism, are a basis for great progress." Also, the EVAC project enabled three young researchers to obtain their PhD. Dr. Manouk Vrieling, who obtained her PhD during EVAC underlines the importance of public–private partnerships in veterinary research. “There are a number of animal diseases, like bovine mastitis, that still need a solution. When multiple partners work together with a company that has the power to carry out large in vivo studies, the step towards a product is smaller, which is a great thing.”
ALTANT Organisation structure

A schematic representation of the ALTANT organisation structure is shown in figure 5 and descriptions of the different tasks and responsibilities of Immuno Valley, the Steering Committee and the Advisory Board can be found in the next paragraphs.

Figure 5. ALTANT Organisation chart

4.1. PROGRAMME MANAGEMENT BY IMMUNO VALLEY
Immuno Valley was founded in 2008 as a network organization consisting of companies and knowledge institutes in human and animal health. The goal of Immuno Valley is to stimulate collaboration and innovation in infectious diseases research in humans and animals. Due to its unique combination of leading pharmaceutical companies and research institutes, Immuno Valley was asked to coordinate the ALTANT programme.

Since 2008, the Immuno Valley network has grown to around 35 partners from multinational pharma, small and medium sized enterprises (SMEs) and knowledge institutes. In the Immuno Valley network, partners from human and animal health can find each other and are actively matched by the Immuno Valley team to build new partnerships with the aim to develop solutions for infectious diseases in animals and man. The Immuno Valley team has the required experience to bridge the gap between science and industry partners. By speaking the language of both academic scientists and people working in international pharma companies, Immuno Valley
was able to coordinate the ALTANT programme in an efficient way. Immuno Valley took an advisory role on, for example, setting realistic goals and making sure that those goals were met. In addition, Immuno Valley was the first contact point for the Ministry and the project teams regarding ALTANT.

Immuno Valley initiated and coordinated ALTANT phase I that resulted in 14 project ideas of which 4 projects were selected and being executed between 2009 and 2010. At the end of phase I, Immuno Valley actively reached out to industry to match companies in the veterinary sector with the ongoing ALTANT projects. The project teams of the four ALTANT projects were supported and advised on presenting the potential application profile of the alternative to antibiotics that was being investigated. The presentations resulted in industrial commitment for all four projects and eventually the continuation of the ASIA and EVAC project into ALTANT phase II.

In ALTANT phase II, Immuno Valley facilitated the public-private collaboration between the academic, industrial and governmental parties involved. Immuno Valley drafted the consortium and project agreements, taking all wishes and demands of all the partners into account. In the end, all partners supported the agreements that were made, leading to efficient and productive collaborations. The Immuno Valley team tracked the progress of the two projects, was responsible for compiling yearly reports of program progress to the Ministry, and was taking the lead in communication and dissemination activities (see paragraph 4.4).

4.2. STEERING COMMITTEE
ASIA and EVAC were directed by a Steering Committee, that was responsible for both the project progress and the achievement of milestones. The Steering Committee had bi-annual meetings in which agreements where made on the direction of the project. The Steering Committee consisted of scientific experts, veterinary pharma market experts, external innovation managers and business developers of the partners in the project and a representative from Immuno Valley as an advisor. The project leaders, Prof. Henk Haagsman and Prof. Jos van Strijp were actively involved in the Steering Committee and biannually reporting of the latest developments of
their projects during the Steering Committee meetings. Jos van Strijp: “The Steering Committee was very important for the changes that we needed to make during the course of the project. The expectations we wrote down in our original project plan four years ago led to different results than expected. We learned that some things just did not work as we thought in advance. The Steering Committee played a central role in changing course, since they should give formal permission to make those big decisions. We learned a lot during the steering committee meetings, not only scientifically but also on creating support for the project.”

4.3. ADVISORY BOARD

The role of the ALTANT Advisory Board, installed by the Ministry of Economic Affairs, was to independently track the programme progress along with consultation of stakeholders working in animal and human health. The Advisory Board consisted of representatives from the Ministry of Economic Affairs, the Royal veterinary association of the Netherlands (Koninklijke Nederlandse Maatschappij voor Diergeneeskunde in Dutch), the Dutch National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu in Dutch) and Immuno Valley. During the course of a long-term research and development programme as ALTANT, planning and focus may change due to changing circumstances and achieved results. This is of course inherent to doing research, but the Advisory Board checked if these changes were still in line with the original project focus of ASIA and EVAC. Tonnie Greutink from the Ministry of Economic Affairs and chair of the Advisory Board commented: “Our role was to control that the government funding was spent well and according to the project outline. We did not discuss project details with the project partners and were not involved in making scientific decisions, but we did oversee the project progress and reporting.” The collaboration with Immuno Valley was also highly appreciated: “Our relationship with Immuno Valley was very strong from the very beginning. Immuno Valley put a lot of effort in building the ALTANT consortium and in making each other stronger.” Advisory Board member Hugo de Groot continued: “ALTANT was a very ambitious programme, and although it did not result in actual products yet, it still can be considered as a very successful programme
since it had a huge spin-off of gained knowledge and the graduation of PhDs.” About Immuno Valley, Hugo de Groot said: “The collaboration with Immuno Valley was great, right from the beginning. Immuno Valley is very involved, enthusiastic, ambitious and driven.”

4.4. ALTANT COMMUNICATION AND DISSEMINATION
The ALTANT progress was actively communicated to the general public by publishing news items about project results and interviews with project leaders (see also Appendix II). In addition, two ALTANT conferences were organised. The ALTANT conferences were aimed at discussing recent scientific advances and offering networking opportunities for participants from academia and industry. Both conferences were a great success, both with over 120 (inter)national participants, and were positively reviewed by the attendants. The participants noted the low threshold to discuss science with the key note speakers and there was ample time for young scientists to present their work in both oral and poster presentations.
Many representatives of human and animal health companies were attending the ALTANT conferences, which stimulated the exchange of ideas between people from academia and industry. The ALTANT conferences resulted in the formation of an international community of experts working in microbiology, immunology and host-pathogen interactions, with an interest in research and development of alternatives to antibiotics that can be involved in future research programmes to this end.
The goal of the ALTANT programme was to develop innovative solutions that could drastically reduce or even eliminate the use of antibiotics in livestock farming, thereby minimizing the risk of antibiotic resistance development. Although no products were delivered that are ready to be developed for application in the veterinary sector, the ALTANT programme did result in important insights that can eventually be translated into therapeutic or preventive interventions for bacterial infections in the future. The scientific and societal impact of ALTANT phase II is shown by more than 25 scientific peer-reviewed international publications, 6 doctoral theses, 2 patent applications, and attention in the media (see Appendix I and II).

The ASIA project focussed on the antibacterial and immunomodulatory properties of host defence peptides (HDPs). It was shown that host defence peptide CATH-2 has antimicrobial activity by permeabilizing, and thereby killing bacteria. In addition, CATH-2 has immunomodulatory properties by improving antigen presentation and increasing the number of phagocytes. Furthermore, a protective effect against bacterial infection when administered in ovo was found. Future plans for ASIA are to continue in unravelling the mechanisms underlying the immunomodulatory capacity of HDPs by means of in vitro and in vivo studies. This should further increase the knowledge how to exploit antimicrobial peptide substrate as alternatives to conventional antibiotics. The EVAC project aimed at developing a vaccine against the evasion molecules of the S. aureus bacterium instead of the bacterium itself. Two evasion molecules were characterized and were shown to indeed evade the immune system by blocking important processes, such as phagocytosis. In order to develop the evasion molecules into an effective vaccine, the most suitable vaccination route and adjuvant were identified. An important finding was that subcutaneous administration near the udder with a vaccine including a potent adjuvant, has potential as a one-shot strategy to increase intramammary antibody responses against bacterial infections. Unfortunately, the experimental vaccine appeared ineffective in in vivo trials, but since a lot of tools were developed that can be used by
other scientists as well, this will benefit the study on evasion molecules and a possible S. aureus vaccine in the future. The scientific findings obtained in the ASIA and EVAC project contribute to a shift in strategies to address the current global antimicrobial resistance issues. For decades, the emphasis of these strategies has been on the development of antibacterial compounds that have a high risk of inducing resistance themselves. By investigating novel therapies based on immunomodulatory agents or vaccines targeting evasion molecules, ASIA and EVAC have contributed to the development of innovative approaches that can lead to a reduction in the use of traditional classes of antibiotics.

In addition to the scientific knowledge that was obtained during ALTANT phase II, important lessons were learned on management of public-private partnerships as well. In order to have a successful collaboration it is important to make transparent agreements and expectations before the start of the project. This not only applies to research goals and milestones but also on project management obligations such as reporting, communication and dissemination deliverables. It has become apparent in the ALTANT programme that an experienced and competent management team composed of representatives of the collaborating partners and dedicated to this task is essential to achieve this. Furthermore, flexibility in the project planning has turned out to be important, since during innovation, the results might force that the initial course needs to be adapted. Such changes, however, need to be thoroughly discussed in order to prevent tension between the public and private partners and to keep track of the initial project goals that were set in the agreement. The organisational set-up in the ALTANT programme with a project steering committee to fulfil this task has proven to be effective. Also the Advisory Board that independently controlled the progress of the ALTANT programme was very important in effective management of this public-private partnership. Tonnie Greutink, chairman of the Advisory board: “ALTANT is an excellent example of a successful collaboration between industry, university and government, and shows that during a research programme, innovation cannot always be 100% successful, but all gained knowledge is valuable, also for other research and future collaborations.”
The ALTANT programme is a perfect example of a successful public-private partnership with an innovative funding model that was attractive for both knowledge institutes and companies and resulted in efficient collaborations in a One Health context. By designing a first phase that was fully financially supported by the government, academic research groups could translate their fundamental research towards a “proof of potential” delivering results that convinced industry to invest in the second phase of ALTANT. Particularly since the government was also willing to partially fund this second phase, the high risk coming along with translational research towards a product profile ready for development, could be shared among the partners. Dr. Paul Vermeij, MSD AH: “Collaboration with a university research group can bridge the knowledge gap that we have in our industry, in which R&D focuses on product development rather than fundamental science. The partnership in EVAC works, since we both bring added value to the project and respect each other’s interests. In this setting collaboration between animal health and human health research groups can be effective.”

During the ALTANT programme, a lot of effort was put in communication and dissemination activities. This effort paid off since a strong community of experts with an interest in research and development of alternatives to antibiotics was built that will also be actively involved in future research programmes. For instance, Immuno Valley is the initiator and coordinator of a new public-private research programme on bacterial vaccines, ‘Bac-Vactory’. Within this research programme, novel vaccine technology platforms will be developed in order to effectively prevent bacterial infections in animals and humans. Similarly to ALTANT, Bac-Vactory is using a multidisciplinary One Health approach where public and private partners work together. Communication and dissemination activities, including conferences for knowledge sharing and networking, will also be organized around the Bac-Vactory programme by which the community of experts that is formed during ALTANT can be further enforced. Another initiative that will benefit from this community is the recently launched Netherlands Antibiotic Development Platform (NADP) supported by the Ministry of Health, Welfare and Sports. NADP facilitates the collaboration between public and private organisations, to accelerate the development
of new antibiotics and alternative therapies for infectious diseases in humans and animals. Immuno Valley will coordinate all communication and dissemination activities of the platform including matchmaking and creating international visibility.

In conclusion, the scientific knowledge and the community that was built during ALTANT phase II does not stop here but will continue to grow in the future. As such, ALTANT contributed successfully to a boost in research and development of alternatives to antibiotics for use in animals and humans.
References


(2) MARAN 2016. Monitoring of antimicrobial resistance and antibiotic usage in animals in The Netherlands in 2015. Report published by Wageningen Bioveterinary Research in collaboration with the Food and Consumer Product Safety Authority (NVWA), the National Institute for Public Health and the Environment (RIVM) and the Netherlands Veterinary Medicines Authority (SDa).


http://www.immunovalley.nl/altant/


Appendix I
Scientific publications and patents

ASIA
Scientific publications

PhD theses
- Dr. Maarten Coorens, Cathelicidins and the regulation of the innate immune system, graduation date: 26 September 2016
- Dr. Tryntsje Cuperus, A tale of chicken cathelicidin-2, graduation date: 21 June 2016
- Dr. Viktoria Schneider, Shedding light on antibacterial activities of cathelicidins, graduation date: 26 May 2016

Patents
EVAC

Scientific publications:


PhD theses:
• Dr. Annemarie Kuijpers, Mechanisms to suppress or enhance phagocytosis of Staphylococci, graduation date: 16 June 2016
• Dr. Eveline Myrthe Boerhout, Intramammary immunity against Staphylococcus aureus in cattle, graduation date: 6 October 2016
• Dr. Manouk Vrieling, Host Adaptation of Staphylococcal Leukotoxins, graduation date: 15 December 2016
Appendix II/
ALTANT in the media

Websites
• ALTernatives to ANTibiotics program page on Immuno Valley website
  http://www.immunovalley.nl/altant/
• ALTANT ASIA project page on Immuno Valley website
  http://www.immunovalley.nl/altant/altant-asia-project/
• ALTANT EVAC project page on Immuno Valley website
  http://www.immunovalley.nl/altant/altant-evac-project/
• ALTANT Conference
  http://ivevents.aneto.nl/altant-conference-2016/

News items, Interviews and press releases
• Businesses and academia work together to develop alternatives to antibiotics in livestock farming,
  Press release Immuno Valley on start of ALTANT phase II
  December 2011
• ‘Merck Partners with Dutch University on Quest for Bovine Mastitis Vaccine’, publication about
  EVAC on the website of Veterinary Practice news
  January 2012
  http://www.veterinarypracticenews.com/January-2012/Merck-Partners-With-Dutch-University-
  On-Quest-For-Bovine-Mastitis-Vaccine/
• Press release MSD Animal Health on EVAC ‘Merck Animal Health Enters Public-Private Partnership
  to Develop Innovative Strategies to Complement Bovine Mastitis Treatment’
  January 2012
• ‘Exploring antibiotic alternatives: farm animals’ natural defences’, publication on ASIA project on
  website of Pig ProgressJune 2012
  http://www.pigprogress.net/Health-Diseases/Health/2012/6/Exploring-antibiotic-alternatives-]
  farm-animals-natural-defences-PP008984W/
• KNAW subsidy for ALTANT conference ‘Innate host defence mechanisms in infections’
  December 2013
  mechanisms-in-infections/
• ‘Immuno Valley; heldere focus op infectieziekten bij mens en dier’, interview with Henk Haagsman
  and Liana Steeghs on ALTANT, in professional journal ‘Immuun’
  December 2013
• ‘Ontwikkeling alternatieven antibiotica zeer moeizaam’, publication in ‘Molkvee’, professional
  journal for veterinarians
  May 2014
  http://edepot.wur.nl/306828
• ‘Stimuleren afweersysteem alternatief voor antibiotica?’, interview with Henk Haagsman about ASIA
  July 2014
  http://pers.uu.nl/stimuleren-afweersysteem-alternatief-voor-antibiotica
• Publication in Journal of Immunology from ALTANT ASIA project
  November 2015
  project/
- KNAW subsidy for ALTANT conference ‘Innate host defence mechanisms and infections’
  December 2015

- ‘Eiwit vervangt antibiotic’, Interview with Tryntsje Cuperus about her PhD defense on ASIA project
  June 2016
  http://www.boerderij.nl/Pluimveehouderij/Nieuws/2016/6/Eiwit-vervangt-antibiotica-2824502W/

- ‘ALTANT ASIA thesis defence: Chicken CATH-2 possible alternative to antibiotics’, news item on
  Immuno Valley website
  September 2016