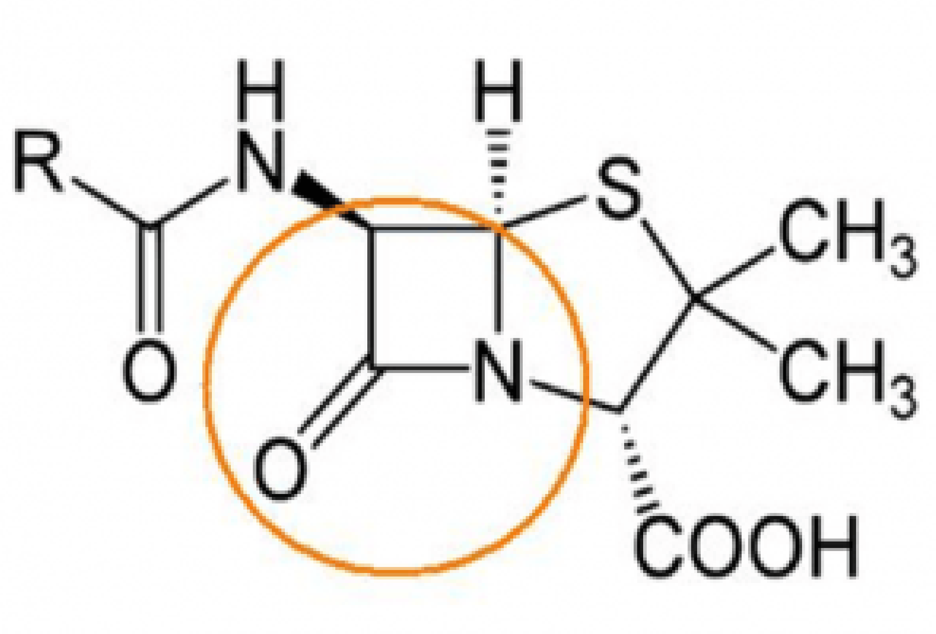
Secondary plant compounds against antibiotic-resistant E. coli

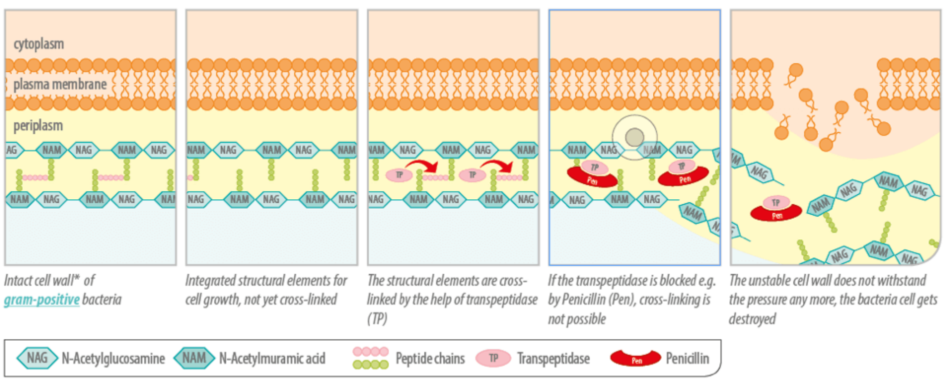
Due to incorrect therapeutic or preventive use of antibiotics in animal production as well as in human medicine, occurrence of antibiotic resistant pathogens has become a widespread problem. Enterobacteria in particular (e.g. Salmonella, Klebsiella, E. coli) possess a special mechanism of resistance. By producing special enzymes (ß-lactamases), they are able to withstand the attack of so-called ß-lactam antibiotics. The genes for this ability (resistance genes) can also be transferred to other bacteria resulting in a continuously increasing problem. Divers point mutations within the ß-lactamase genes lead to the occurrence of „Extended-Spectrum-Beta-Lactamases“ (ESBL), which are able to hydrolyse most of the ß-Lactam-antibiotics. AmpC Beta-Lactamases (AmpC) are enzymes, which express a resistance against penicillins, cephalosporins of the second and third generation as well as cephamycins.

What are ß-lactam antibiotics?
The group of ß-lactam antibiotics consists of penicillins, cephalosporins, monobactams and carbapenems.
A characteristic of these antibiotics is the lactam ring (marked in orange):



Mode of action of ß-lactam antibiotic
If a bacterial cell is growing, the cell wall also has to grow. For this purpose, existing conjunctions are cracked and new components are inserted. ß-lactam-antibiotics disturb the process of cell wall construction by blocking an enzyme needed, the transpeptidase. If crosslinks necessary for the stability of the cell wall cannot be created, the bacteria cannot survive. Resistant bacteria, which are able to produce ß-lactamases, destroy the ß-lactam antibiotics and prevent their own destruction.

Secondary plant compounds
Secondary plant compounds and their components are able to prevent or slow down the growth of moulds, yeasts, viruses and bacteria. They attack at various sites, particularly the membrane and the cytoplasm. Sometimes they change the whole morphology of the cell. In the case of gram-negative bacteria, secondary plant compounds (hydrophobic) have to be mixed with an emulsifier so that they can pass the cell wall which is open only for small hydrophilic solutes. The modes of action of secondary plant compounds depend on their chemical composition. It also depends on whether single substances or blends (with possible positive or negative synergies) are used. It has been observed that extracts of spices have a lower antimicrobial efficacy than the entire spice.



*For simplicity, only two layers of peptidoglycans (NAM-NAG) are shown. Normally the cell wall consists of many layers.

The best explained mode of action is the one of thymol and carvacrol, the major components of the oils of thyme and oregano. They are able to incorporate into the bacterial membrane and to disrupt its integrity. This increases the permeability of the cell membrane for ions and other small molecules such as ATP leading to the decrease of the electrochemical gradient above the cell membrane and to the loss of energy equivalents of the cell.

Trial (Scotland)

Design

Two strains of ESBL-producing and AmpC respectively, isolated from the field, a non-resistant strain of E. coli as control. Suspensions of the strains with $1×10^4$ KBE/ml were incubated for 6-7 h at 37°C together with different concentrations of Activo Liquid or with cefotaxime, a cephalosporin. The suspensions were then put on LB-Agar plates and bacteria colonies were counted after a further 18-22h incubation at 37°C. Evaluation of the effects of Activo Liquid on ESBL-producing as well as on E. coli resistant for aminopenicillin and cephalosporin (AmpC)

Results
The antimicrobial efficacy of the blend of secondary plant compounds depended on concentration with bactericidal effect at higher concentrations and bacteriostatic at dilutions up to 0,1%. It is also possible that bacteria could develop a resistance to secondary plant compounds; the probability is however relatively low, due to the fact that essential oils contain hundreds of chemical components (more than antibiotics) making it difficult for bacteria to adapt.

Phytogenics can positively influence the efficacy of antibiotics

Many veterinary antibiotics are applied via the waterline, where they are dosed in combination with other feed additives. Amongst those are mixtures of secondary plant compounds with a proven antimicrobial efficacy against veterinary pathogenic bacteria. However, little research has been done to evaluate any effect that antibiotics and phytogenics may have on each other. A possible influence of phytogenics on the efficacy of antibiotics through the combined administration would require a change in application recommendations of antibiotics and phytogenic feed additives. In the case of no interaction, no changes would be necessary. If they were to interact in a positive way, the dosages could be lowered and if they interact in a negative way, a combined application would be avoided.

Antibiotics and SPC's in co-incubation

There are different groups of antibiotics depending on the chemical structure and on the pathogen they target. Some impair the cell wall or the cytoplasmic membrane (polymyxins, ß-lactam antibiotics) and some affect protein synthesis (macrolides, Chloramphenicol, Lincospectin, tetracyclines, aminoglycosides). Others compromise DNA and RNA synthesis (fluorchinolones, ansamycines) and some disturb the metabolism of e.g. folic acid (Trimethoprim).

The intention of a trial with these different groups of antibiotics was to evaluate possible interactions they may have with a combination of secondary plant compounds. Four ESBL producing *E. coli* field isolates from poultry flocks were experimentally assessed as well as a ß-lactamase positive and a ß-lactamase negative reference strain as quality control strains for antimicrobial susceptibility testing.

Two-fold serial dilutions of antibiotics and the liquid product based on secondary plant compounds were co-incubated in a checkerboard assay. The highest concentration of the antibiotic was chosen according to CLSI standard recommendations. The control of the serial dilution of SPC's was made without antibiotics and vice versa.

Lowering the antibiotic dosage by the use of SPC's

In the experiment all field isolates proved resistant against the ß-lactam antibiotics, two field isolates and one reference strain were resistant against tetracyclines and macrolides and one field isolate and one reference strain against aminoglycocides.

The results showed that there was no negative influence of the antibiotics on the SPC's and vice versa. Moreover, for several classes of antibiotics an additive to synergistic effect was observed to such an extent that an antibiotic effect could be achieved with half or even one quarter of the former effective dosage. The dosage of the SPC-mixture could also be reduced. Based on the results of this *in vitro* experiment it can be stated that in the case of antibiotic resistance, the option exists to apply a phytogenic product with broad antimicrobial efficacy. Even more, for most combinations between antibiotics and Activo Liquid, a defined mixture of secondary plant compounds, their combined use potentiates the individual efficacy of either compound class against *E.coli* strains *in vitro*. This adds further benefits to the improvements in animal performance and health, for which a number of phytogenic feed additives have already proven effective.

A powerful alternative to antibiotics for Aquaculture

Global aquaculture has grown dramatically and shrimp cultivation areas, in particular, have expanded. Unfortunately, the shrimp industry, in particular, faces major problems with bacterial diseases. One of the most important diseases in shrimp is vibriosis. Mortality rates of up to 100 % are possible, so economic losses can be devastating.

Characteristics of vibriosis
Vibriosis generally occurs in all life stages, but mainly in hatcheries. Vibrios are found as normal flora in the hepatopancreas of the healthy crustacean. They can turn from tolerated to pathogenic, if environmental conditions are compromised: e.g. over / underfeeding, overcrowding or decreased levels of oxygen.
The animals can be infected orally, through wounds in the exoskeleton or pores, the gills or the midgut. There are different expressions of the disease depending on which parts of the animal are affected (e.g. appendage and cuticular vibriosis).

Negative effects on the ecology
As antibiotics for shrimps are applied orally together with the feed, not all of them reach their target. An estimated 15-40 % are not ingested due to feeding falling to the bottom. A fraction of the ingested antibiotics is also not absorbed in the body and is excreted. All of these antibiotics stay in the water or sink to the bottom. The number of antibiotics that remain in the water or sediment varies from 1 % (chloramphenicol) up to 90 % (Oxytetracycline).
It is estimated that 70-90 % of antibiotics used in the therapy of farmed organisms end up in the environment and sediment and lead to the development of antibiotic resistance.

Secondary Plant Compounds (SPCs) – a good tool to reduce the use of antibiotics?
SPCs and their components are able to slow down or prevent the growth of molds, viruses, and bacteria. They impair them by acting at different parts/mechanisms of the cells (e.g. cell membrane, transport systems, cell contents, flagella development, quorum sensing…). The best-explained mode of action is one of thymol and carvacrol extracted from thyme and oregano. These substances are able to penetrate the bacterial membrane and disrupt its integrity causing loss of ions or energy equivalents.

Several trials conducted show the high efficacy of secondary plant compounds in aquaculture.

1. Scientific Trial (Kasetsart University, Thailand)

Design

a) 4 groups (6 replicates each) of White Leg Shrimp (L. vannamei) were housed in 100 L aquaria with 10 animals each.

Control: Standard feed, no additive
AB-Group: Standard feed + 10 ppm Enrofloxacin
Activo Group 1: Standard feed + 100 g Activo/t of feed
Activo Group 2: Standard feed + 200 g Activo/t of feed

Evaluation of mortality and specific growth

a) End of the feeding trial: stressing of the shrimp (high water temperature, 33°C for 1 hour), then challenge with Vibrio parahaemolyticus (7,6 x 106 cfu/ml) by subcutaneous injection.

Evaluation of mortality

a) Survival rates in the AB-Group (93,3 %) and in the Activo Group 2 (90,0 %) were similar. The specific growth rate of the AB-Group (2,32 %/day) and the Activo Group 2 (2,22 %/day) were higher than the control (1,94 %/day). The Activo Group 1 (2,18 %/day) ranged performance-wise between the control and AB-Group.

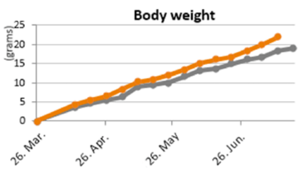
b) After the challenge, mortality in the control group (43,3%) was approximately twice as high as in the AB-Group (20 %) and in the two Activo groups (both 23,3%).

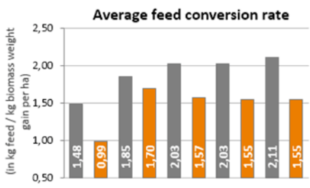
2. Field Trial (Shrimp farm Ecuador)

Design
Two ponds with 80.000 shrimps/ha
Control (3 ha): standard feed
Activo Aqua Group (5 ha): standard feed + 2 kg Activo Aqua (Activo upgraded by immune system stimulating- mannan-oligosaccharides) /t of feed on top

Evaluation of average shrimp weight at regular intervals

Results
Activo Aqua Group showed a consistently better development of body weight compared to the control, resulting in a shorter cultivation period until harvesting (112 compared to 123) and therefore a higher turnover of animals. Feed conversion in the Activo Aqua group was better in every growth stage.





Both trials present secondary plant compounds as a good alternative to antibiotic growth promoters. In the case of disease, they decrease mortality. Under standard conditions, the improved development shortens time to harvest and increases the turnover. The improved feed conversion lowers feeding costs.