

# Phytomolecules: A tool against antibiotic-resistant *E. coli*



## Diseases caused by *E. coli* entail use of antibiotics in animal production

*E. coli* infections are a major problem in pig production. Especially young animals with an incompletely developed immune system are often unable to cope with the cavalcade of pathogens. In poultry, *E. coli* are responsible for oedema, but also for [respiratory diseases](#). In young piglets, *E. coli* cause diarrhoea, oedema, endotoxic shock and death. In order to cure the animals, antibiotics often must be applied. Besides this curative application, antibiotics were and in many countries still are used prophylactically and as growth promoters.

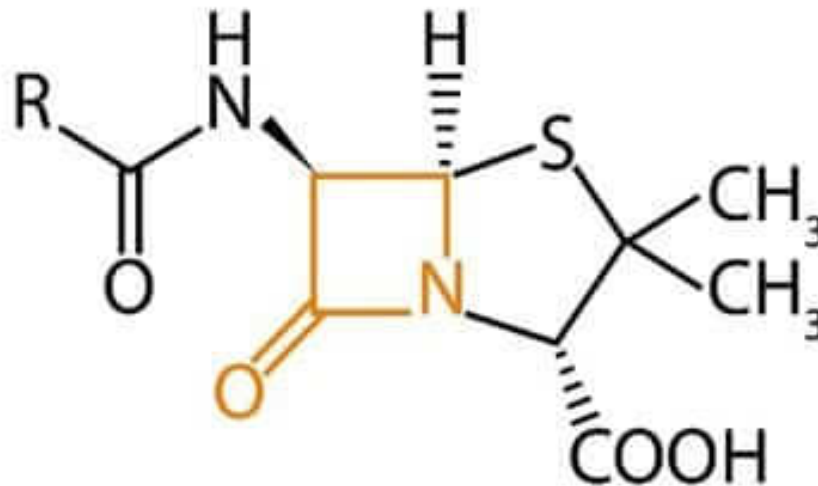
The excessive use of antibiotics, however, leads to the [occurrence of antimicrobial resistance](#) (AMR): due to mutations, resistance genes are created which enable enterobacteria such as *Salmonella*, *Klebsiella* and *E. coli* to produce enzymes ( $\beta$ -lactamases) in order to withstand  $\beta$ -lactam antibiotics. In case of an antibiotic treatment, the resistant bacteria survive whereas the other bacteria die.

The major problem here is that these resistance genes can be transferred to other bacteria. Harmless bacteria can thus transfer resistance genes to dangerous pathogens, which then cannot be combatted with antibiotics anymore. In this article we explore in detail how AMR happens and how phytomolecules, which have antimicrobial properties, could be a key tool to reduce the need for antibiotics in animal production.

## How $\beta$ -lactam antibiotics work

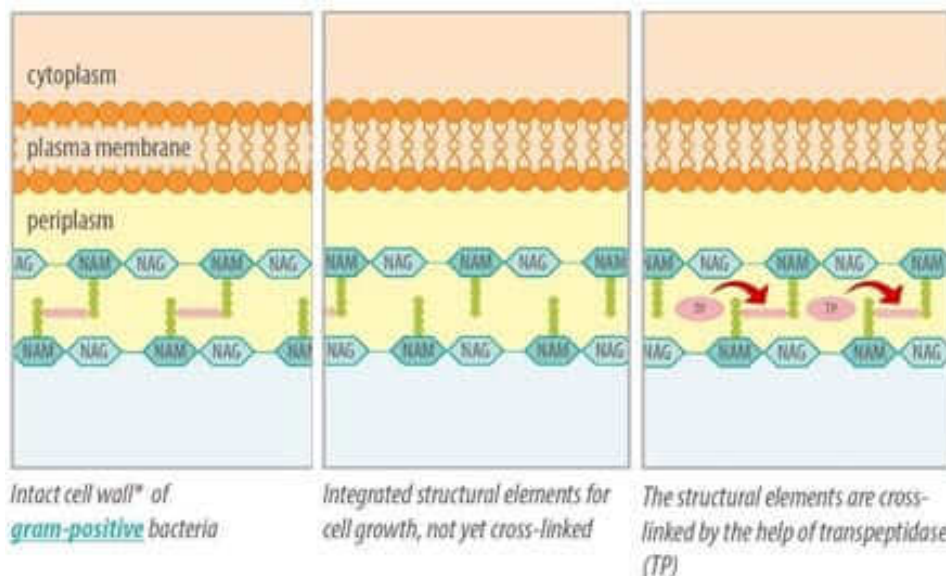
The group of  $\beta$ -lactam antibiotics consists of penicillins, cephalosporins, monobactams, and carbapenems. These antibiotics are characterised by their lactam ring (Figure 1).

Figure 1: An antibiotic with a  $\beta$ -lactam ring (in orange)



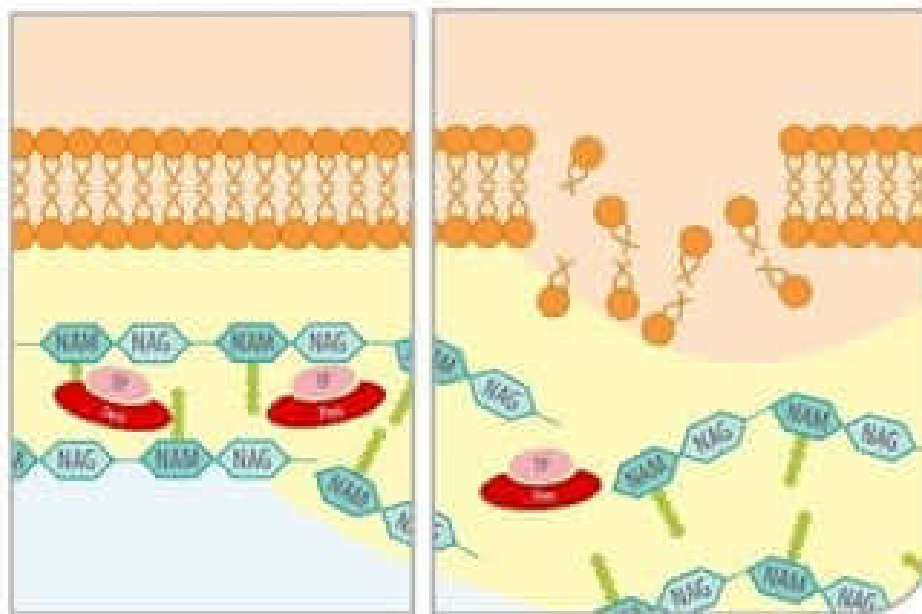
If bacteria are growing, the cell wall also has to grow. For this purpose existing conjunctions are cracked and new components are inserted. In order for the cell wall to remain a solid barrier, the new components must be interconnected by crosslinks. For the creation of these crosslinks an enzyme is essential, the transpeptidase (figure 2).

Figure 2: building up a stable cell wall with the help of transpeptidase



Due to their structure,  $\beta$ -lactam-antibiotics also fit as binding partner for transpeptidase. They bind to the enzyme and block it (Kohanski et al., 2010). The crosslinks cannot be created and the stabilization of the cell wall is prevented. Disturbance of cell wall stability leads to the death of the bacterial cell, hence  $\beta$ -lactam antibiotics act bactericidal.

Figure 3: blocked by  $\beta$ -lactam antibiotics, transpeptidase cannot serve as enzyme for building the cell wall



*If the transpeptidase is blocked e.g. by Penicillin (Pen), cross-linking is not possible*

*The unstable cell wall does not withstand the pressure any more, the bacteria cell gets destroyed*

## The challenge: *E. coli* producing $\beta$ -lactamases

Resistant bacteria, which are able to produce  $\beta$ -lactamases – enzymes that destroy the  $\beta$ -lactam ring – prevent their own destruction. Divers point mutations within the  $\beta$ -lactamase genes lead to the occurrence of “extended-spectrum-beta-lactamases” (ESBL). ESBL are able to inactivate most of the  $\beta$ -Lactam-antibiotics.

Another mutation leads to so-called AmpC (aminopenicillin and cephalosporin)  $\beta$ -lactamases. They enable the *E. coli* to express a resistance against penicillins, cephalosporins of the second and third generation as well as against cephamycins.

## Phytomolecules – an alternative?

One approach to reduce the use of antibiotics is the utilization of phytomolecules. These secondary metabolites are produced by plants to protect themselves from moulds, yeasts, bacteria and other harmful organisms.

The use of plants and their extracts in human and veterinary medicine is well-established for centuries. Besides digestive and antioxidant characteristics they are well known for their bacteriostatic and bactericidal effects.

Consisting of a high number of chemical compounds, they attack at diverse points and their antimicrobial effect is not caused by only one single specific mechanism. This is crucial because it is therefore very unlikely that bacteria can develop resistances to phytomolecules like they do to antibiotics.

## How phytomolecules work

Mostly, phytomolecules act at the cell wall and the cytoplasm membrane level. Sometimes they change the whole morphology of the cell. This mode of action has been studied extensively for 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 the energy carrier ATP

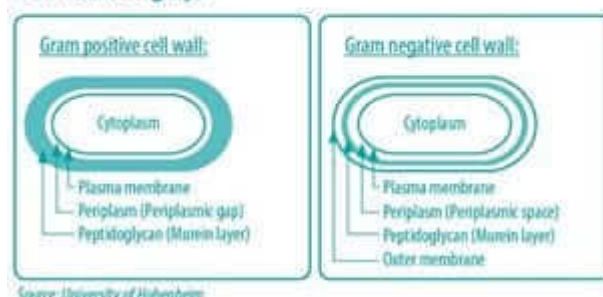
(Adenosin-tri-phosphate). It leads to the decrease of the electrochemical gradient above the cell membrane and to the loss of energy equivalents of the cell.

### A special challenge: gram-negative bacteria

Gram-negative bacteria such as *E. coli* and *Salmonella* pose a special challenge. The presence of lipopolysaccharides in the outer membrane (OM) provides the gram-negative bacteria with a hydrophilic surface ([Nikaido, 2003](#); [Nazarro et al., 2013](#)) (see also blue infobox).

**Gram-positive bacteria and gram-negative bacteria:**  
Bacteria differ in the construction of their cell walls. The Danish bacteriologist Hans Christian Gram (1853–1938) developed a colouring method to differentiate the bacteria. It is not possible to assign all bacteria to both groups. There are also gram-variable and gram-indefinite species.

#### Cell wall (roughly):



The cell wall therefore only allows the passage of small hydrophilic solutes and is a barrier against macromolecules and hydrophobic compounds such as hydrophobic antibiotics and toxic drugs. The bypassing of the OM therefore is a prerequisite for any solute to exert bactericidal activity toward gram-negative bacteria (Helander et al., 1998).

Based on their trial results Helander et al. (1998) (1998) concluded that trans-cinnamaldehyde and partly also thymol and carvacrol gain access to the periplasm and to the deeper parts of the cell. Nikaido (1996) also concluded that OM-traversing porin proteins allow the penetration of lipophilic probes at significant rates.

## Evaluating phytomolecules I - in vitro trial, Scotland

A trial conducted in Scotland evaluated the effects of Activo Liquid, a mixture of selected phytomolecules and citric acid, on ESBL-producing *E. coli* as well as on *E. coli* that generate AmpC.

### Material and methods

For the trial two strains for each group were isolated from the field, a non-resistant strain of *E. coli* served as control. Suspensions of the strains with  $1 \times 10^4$  CFU/ml were incubated for 6-7 h at 37°C (98.6°F) together with diverse concentrations of Activo Liquid or with cefotaxime, a cephalosporin. The cefotaxime group served as a control for differentiating resistant and non-resistant *E. coli*.

The suspensions were put on LB agar plates and bacteria colonies were counted after further 18-22h incubation at 37°C.

### Results

The antimicrobial efficacy of the blend of phytomolecules depended on the concentration at which they were used (see table 1). A bacteriostatic effect could be shown at dilutions up to 0.1 %, a bactericidal effect at higher concentrations.

Table 1: Effect of phytomolecules against resistant *E. coli* producing ESBL and AmpC in poultry

| Poultry Microbiology<br>Laboratory, Edinburgh,<br>Scotland             | Cefotaxime | Phytomolecules (Activo® Liquid) |       |       |       |
|--|------------|---------------------------------|-------|-------|-------|
|  | 30 µg / ml | 0.1 %                           | 0.2 % | 0.4 % | 0.5 % |
| <i>E. coli</i> ESBL 1 (Poultry)  | -          | +                               | ++    | ++    | ++    |
| <i>E. coli</i> ESBL 2 (Poultry)  | -          | +                               | ++    | ++    | ++    |
| <i>E. coli</i> AmpC 1 (Poultry)  | -          | +                               | ++    | ++    | ++    |
| <i>E. coli</i> AmpC 2 (Poultry)  | -          | ++                              | ++    | ++    | ++    |
| <i>E. coli</i> non-resistant   | +          | +                               | ++    | ++    | ++    |
| - no effect<br>+ growth inhibiting (bacteriostatic)<br>++ bactericidal |            |                                 |       |       |       |

## Evaluating phytomolecules II - in vitro trial, Germany

A further trial was conducted in Germany (Vaxxinova, Münster), confirming the preceding results.

### Material and methods

Four ESBL producing *E. coli* all isolated from farms and a non-resistant reference strain as control were tested concerning their sensitivity against Activo Liquid. Every bacteria strain (Conc.:  $1 \times 10^4$  CFU/ml) was subjected to a bacterial inhibition assay in an appropriate medium at 37°C for 6-7 hours.

### Results

In this trial Activo Liquid also showed a dose-dependent efficacy, with no or just a bacteriostatic effect up to a concentration of 0.1 %, but bactericidal effects at a concentration of  $\geq 0.2$  % (table 2).

Table 2: Effect of phytomolecules against resistant ESBL producing *E. coli* in pig and in poultry

| Vaxxinova GmbH,<br>Münster   | Phytomolecules (Activo® Liquid) |       |       |     |
|--|---------------------------------|-------|-------|-----|
|  | 0.1 %                           | 0.2 % | 0.4 % | 1 % |
| <i>E. coli</i> ATCC25922   | +                               | ++    | ++    | ++  |
| <i>E. coli</i> ESBL 1 (Pig)  | -                               | ++    | ++    | ++  |
| <i>E. coli</i> ESBL 2 (Pig)  | +                               | ++    | ++    | ++  |
| <i>E. coli</i> ESBL 3 (Poultry)  | +                               | ++    | ++    | ++  |
| <i>E. coli</i> ESBL 4 (Poultry)  | -                               | ++    | ++    | ++  |
| - no effect<br>+ growth inhibiting (bacteriostatic)<br>++ bactericidal |                                 |       |       |     |



## Phytomolecules: a promising outlook

*E. coli* infections have devastating effects on animals, from diarrhea to edema, enterotoxic shock and even death. Antibiotic treatments have long been the only practicable answer. However, their excessive use– for instance, the metaphylactic application to thousands of animals in a flock– has led to the development of resistant strains. There is evidence that a reduction of antibiotic use reduces the occurrence of resistances ([Dutil et al., 2010](#)).

The results of the two in vitro trials in Scotland and Germany demonstrate the bactericidal effects of [phytomolecules](#) on *E. coli* that produce ESBL and AmpC. Using phytomolecules could thus reduce the use of antibiotics and therefore also the occurrence of AMR.

While it is theoretically possible for bacteria to also become resistant against phytomolecules, the probability of this happening is very low: unlike antibiotics, phytomolecules contain hundreds of chemical components with different modes of action. This makes it exceedingly difficult for bacteria to adapt and develop resistance. To tackle the problem of antibiotic-resistant *E. coli*, antimicrobial phytomolecules therefore offer a promising, sustainable and long-term solution.

By Dr. Inge Heinzl, Editor, EW Nutrition

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### Literature

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