

## **Development of a competitive exclusion product for chicken meeting the regulatory requirements for registration in the EU**

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### **Introduction**

The animal production in the EU faces a very difficult situation, due to the ban of some feed antibiotics, the foreseeable total ban within the near future and the understandable consumer objections to their intensive use.

Commercially produced poultry lack the natural contact between chicks and mother hens, thus providing them the natural infection stress and triggering of their immune system. As a consequence day old chicks that do not establish a protective micro flora immediately after hatching are susceptible to pathogen colonisation, with special reference to *Salmonella*. Competitive exclusion (CE) is a promising method to protect chicks from infections with pathogenic bacteria. Inoculation of day old chicks with undefined micro flora derived from adult chicken is an effective method for protection from pathogen invasion. In particular the CE concept is able to increase the colonisation resistance of day old chicks against *Salmonella*, besides other food borne pathogens, the most important bacterial contaminant transmitted by poultry. It causes both food borne disease in man and heavy economic losses in the poultry industry.

### **Material and methods**

A project – funded by the European Commission - was designed for the development of a defined multi-species CE product, which in contrast to non-defined products available will meet the requirements for registration in the European Union.

This product is intended to increase food safety, enforce quality measures in agricultural structures and improve quality management in the animal production sectors.

The product development was focussed to control the problems associated with increased occurrence of infectious disease due to the ban of antibiotics (e.g. performance losses in animal husbandry, food borne disease in humans, increased use of therapeutic antibiotics...) and on the other hand to meet the requirements in the European Community to guarantee safety in animal production (Commission Directive 94/40) and safe animal derived products, free of contaminants (e.g. antibiotics, hormones, toxins...). Especially the poultry industry deals with this problem, particularly with the problem of transfer of pathogens (*Salmonella*, *Campylobacter*, *E. coli*) from chickens to humans.

#### ***Isolation and characterization of strains***

Isolation of strains was performed out of healthy chicken of various ages. Pure cultures of isolates were stored for investigations and characterized by microbiological, biochemical and molecular-biological methods.

Screening on functional purposes was performed by inhibition tests like Agar-Diffusion test, adhesion testing in cell culture models, physiological testing and determination of growth characteristics as well as sequencing.

### ***Safety assessment***

Quantitative determination of resistance (MIC determination)

Minimal inhibitory concentration (MIC) of various antimicrobial agents was determined for the potential probiotic candidates. The selected product strains and at least one backup-strain were tested for their antibiotic susceptibilities to 14-15 antibiotics representing a spectrum of clinically relevant antibiotics of all antibiotic classes (SCAN, 2003).

Conjugative transferabilities of vancomycin and tetracycline resistances were studied by direct plate colony (DPC) mating using appropriate recipients, as recommended by SCAN (SCAN, 2003). The procedure was performed according to the protocol of Broadbent and Kondo (1991) [with slight modifications].

Tetracycline and/or vancomycin resistant strains were screened for the presence of the most concerning transferable resistance genes, which were selected based on literature survey.

Polymerase chain reactions were used for detection of vanA and tet(M) genes. DNA from *E. faecalis* (vanA+) and *L. plantarum* (tet(M)+) was used for positive control, and distilled water for contamination control.

### **Test kit development**

The applicability of test kits based on methods like FISH (fluorescence in-situ hybridisation) and qPCR had to be determined prior to development of suitable test systems. The effect of the feed matrix as well as influence of intestinal material on test performance had to be worked out and protocols had to be adapted in order to allow for valid test performance.

### ***Results and discussion***

All strains were normal constituents of the natural intestinal flora of healthy chicken, thus providing a rationale for their safe use as in-feed additives for chicken.

It was shown, that several species were dominant members of the beneficial microflora in the chicken gastrointestinal tract. Their predominance in different intestinal compartments was demonstrated by DGGE.

The final product strains have further been tested for their fermentative characteristics and were found to be complementary regarding their ability to metabolize the most common carbohydrate compounds.

### **Test Kit development**

The industrial interest in the combined use of these specific strains as feed additive led to the requirement for accurate quality control of the product. FISH test kits were developed and subsequently tested on feed samples as well as intestinal samples. The FISH test gave good signals even for encapsulated product.

### **Risk assessment**

As the product as a feed additive for broiler production is intended for entering the food-chain, it was of utmost importance to perform safety assessment prior to in-vivo application.

Antibiotic resistance was tested by various methods, known resistances were assessed for their transferability by conjugation test as well as plasmid isolation. Protocols had to be adapted as there is still a lack of acknowledged methods to ensure extended risk assessment.

Those resistances that were found could be shown to be intrinsic and therefore are very unlikely to be transferred.

## **Efficacy testing**

### **Adhesion testing with cell culture**

For our studies it was important to investigate adhesion ability of the isolates for further selection and to get information about the quantity of adhesion possible. Therefore two tissue culture test systems were established.

Results received from the standard adhesion assay clearly show the ability of the isolates to attach to intestinal cells in vitro. The amount of attached cells varies approximately between  $1.0E+05$  and  $1.0E+06$  cells per  $cm^2$  monolayer surface. We found good correlation for adhesion results between the two cell lines for most of the isolates and repeated testing showed good reproducibility. Additionally it could be demonstrated that some strains adhere to a higher extent to the monolayer and results indicate that the extent of adhesion does not necessarily depend on the genus of the tested strains because there are some differences in between the same species. Testing of some probiotic mixtures resulted in very useful information about the effectiveness of adhesion and it could be demonstrated that supplying probiotic products also results in substantial adhesion although the bacterial strains were supplied as freeze dried products.

Due to the findings of our in vitro investigations the property of adhesion was used as additional selection criteria for bacterial isolates in the course of the development of a probiotic poultry product.

### **Feeding trials**

Several feeding trials have been conducted in a preliminary stage of the product development to test for general applicability and practicability of different feeding strategies (drinking water application, feed application, combined strategies). It turned out, that a combined application is the most efficient way to enhance chicken performance towards increased protection against pathogenic invasion. It is of utmost importance to start the application right after hatching and several settings have been performed to supply chicks during transport from hatchery to the producer with a suitable dosage of probiotic product for improved protection.

In optimized settings at University trials it is always difficult to obtain significance in performance parameters. Once the product formulation is optimized towards a stable product by micro encapsulation, challenge trials will be performed to show the protective potential of the competitive exclusion product in vivo.

In a spontaneous field application with 52.000 chicks it could be demonstrated that performance parameters improved to a remarkable extent in comparison to previous production cycles. For the producer such convincing data are as important for supporting the market with alternatives to antibiotic growth promoters for the future. Legal opportunities have to be provided to cope with the challenging situation in Europe once the ban of antibiotic growth promoters is active.