The Effect of *Bacillus subtilis* Strain on Intestinal Barrier Function and Inflammatory Responses

Gut health is a term used by many, but how can it be quantified and influenced? What is clear is that since the ban on antibiotic growth promoters (AGPs), the occurrence of digestive disturbances in poultry has increased. In addition to their antimicrobial effects, AGPs have been shown to also have an anti-inflammatory effect. That is why any alternative solutions should also be able to prevent inflammatory responses.

Probiotics can be used as additives in order to improve gut health, balancing the gut microbiota and optimising the host inflammatory response. New research has shown that a specific *Bacillus subtilis* strain is able to reduce inflammatory responses by modulating the NF-κB signalling pathway, as well as improving intestinal barrier integrity. This information can now be used by nutritionists to make informed choices about the products they add to poultry feed in order to promote gut health and therefore animal performance.

The Gut Health Balance

Gut health can be defined as the ability of the gut to perform normal physiological, homeostatic and protective functions; thereby supporting its ability to endure various stressors. This definition incorporates the following interacting components: host nutrition, the immune system, intestinal microbiome population and intestinal integrity (Figure 1). If there is an imbalance in these interactions, inflammation will occur – leading to 'leaky gut' (increase of the permeability of the intestinal barrier) and other gut-related disorders.

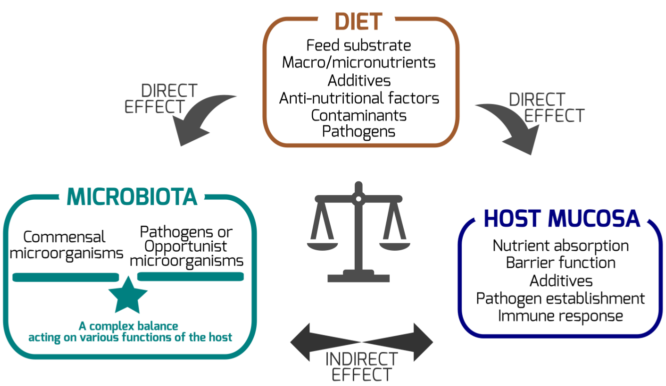

Figure 1. The balance of gut health

The interactions between the microbiota, mucosal barrier, immune system and redox balance have recently been proposed for healthy, pre-disease and disease states[1]. The research concluded that 'the fitness of the host depends on and cannot be seen as separate from its microbiota'. The best illustration is in the case of dysbiosis, which is defined as an undesirable alteration of the microbiota. This in turn affects the host–microbe interactions, essentially leading to inflammation[2] (Figure 2).

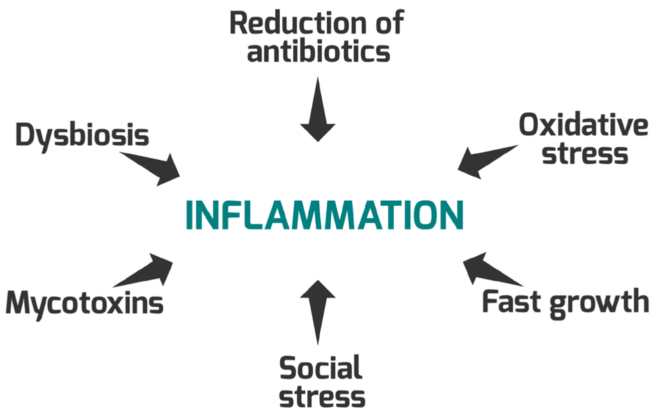

Figure 2. Factors influencing inflammation – including dysbiosis

Use of AGPs and their Mode of Action

Antibiotics have been used for a long time in animal production, for different purposes: as treatments for infectious diseases, prophylactically to prevent disease and as growth promoters. Those antibiotics used as growth promoters, are fed continuously at low (subtherapeutic) levels with the aim of improving performance. They benefit factors, including flock health, uniformity and intestinal microbiota balance, manage digestive disturbances or subclinical challenges, and finally improve economic results for poultry producers.

The mode of action of AGPs has never been fully explained. However, when they are excluded from the diet, bacterial overgrowth often occurs. This infers that, either directly or indirectly, their antimicrobial activity is essential for their beneficial effects.

Prof. Theo Niewold introduced the concept that the anti-inflammatory activity of AGPs is responsible for their beneficial effects, at least as much as their antimicrobial properties[3]. This was suggested predominantly due to the sub-therapeutic concentrations used, as well as the absence of a relationship between antibiotic activity and antibiotic spectrum. This hypothesis is backed up by the fact that AGPs work similarly, in different species with widely divergent microbiota composition. The research also discovered that all antibiotics used as AGPs also have anti-inflammatory properties (Table 1).

Table 1 - Anti-inflammatory and growth promoting effects

Effect of different types of antibiotics

Type of antibiotic	Anti-inflammatory	Used as AGP
Beta-lactams	X	X
Cyclines	V	V
Quinolones	X	X
Macrolides	V	V
Peptides (e.g. Zn-Bacitracin)	V	V

Adapted from Niewold 2007

Table 1. Anti-inflammatory and growth promoting effects of antibiotics

Regulatory Evolution – Ban of AGPs

Many countries have restricted or even banned the use of antibiotics as feed additives, including the EU in 2006.

Where their use is restricted, in order to ensure health and performance, poultry producers have had to develop their management techniques. And for almost 15 years, nutritionists and feed additive manufacturers have been developing alternative solutions. Some of these alternatives, such as probiotics, are now well established in animal nutrition. Their anti-inflammatory and anti-pathogen properties have been widely studied and are of great interest to producers. Probiotics have been shown to prevent and/or restore gut health disorders[4].

Probiotics

Probiotics are live bacteria, which, when delivered at an adequate concentration, confer a beneficial physiological effect on the host[5]. They are used to improve intestinal microbiota balance – thus creating an optimum environment for digestion and nutrient absorption. These beneficial bacteria also help to reduce the growth and/or the pathogenicity of undesirable bacteria; in particular, through the production of antimicrobial compounds such as acids and bacteriocins. Probiotics can also positively influence the immune system thanks to their immunomodulatory properties. Together, these effects lead to better animal health and performance. Whilst antibiotics have a specific effect on pathogens and may also show some anti-inflammatory properties, probiotics have multiple modes of action and global benefits for the host.

The most common microorganisms used as probiotics in animal nutrition include: *Bacilli, Lactobacilli, Bifidobacteria, Enterococci* and yeasts. In this article, data from trials carried out with a unique spore forming *Bacillus subtilis* (strain DSM 29784) are presented. This poultry probiotic solution (Alterion®) has been specifically designed to deliver consistency as well as an optimal activation in the small intestine. *In vivo* trials were carried out at independent

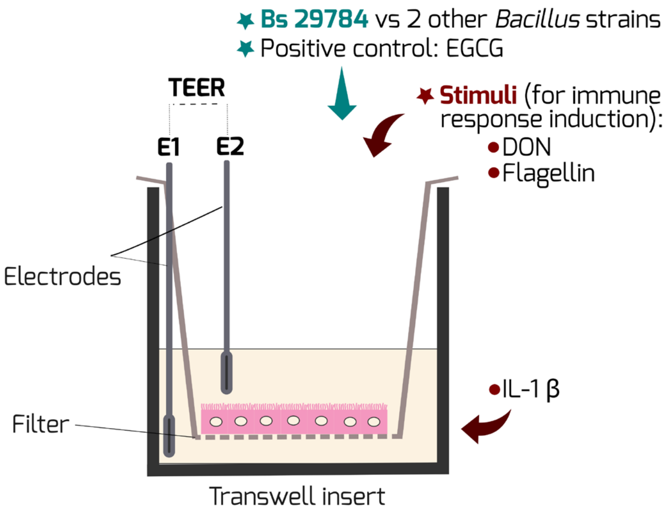

Figure 3. Caco-2 cell model established as a monolayer in a transwell system

scientific institutes, universities and CERN (Centre of Excellence and Research in Nutrition – Adisseo). These were supported by *in vitro* research at Adisseo and Novozymes laboratories, where experiments were first conducted to screen and then further to characterise this unique strain.

Inflammatory Responses Examined

This article evaluates the effect of *Bacillus subtilis* (strain DSM 29784) on intestinal barrier function and inflammatory response.

The Ability of *Bacillus subtilis* DSM 29784 (Bs 29784) to Reduce Inflammatory Response

As a first step, the direct effect was investigated by using an *in vitro* model. Bs 29784 was compared with two other

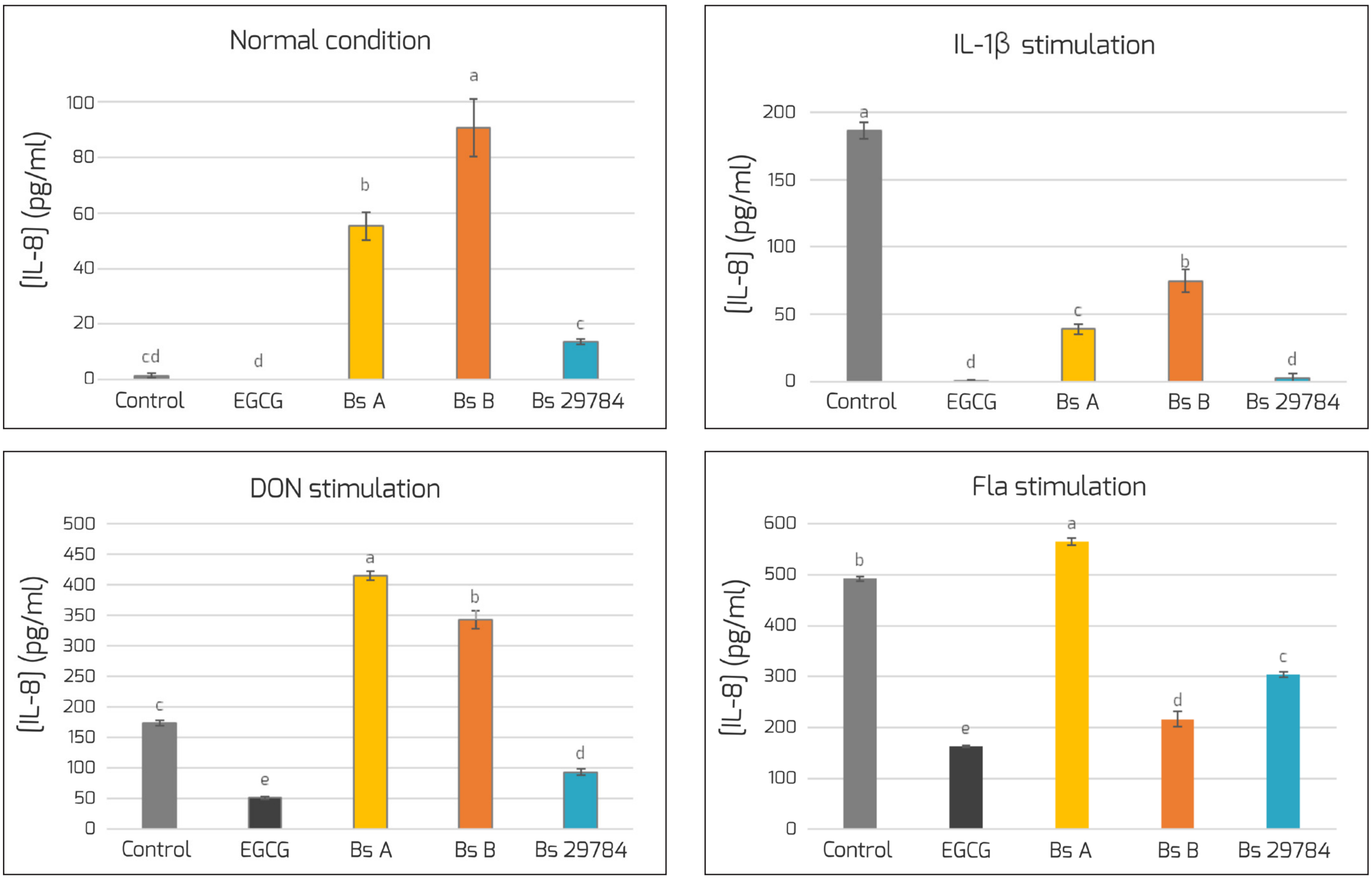

Figure 4: Effect of different Bacillus subtilis strains on IL8 release in standard and challenging conditions

commercially available *B. subtilis* strains (Bs A and Bs B), which are used as probiotics in poultry feed. Epigallocatechin gallate (EGCG), known for its strong anti-inflammatory properties, was used as a positive control (Figure 3).

The human colonic epithelial cell (Caco-2), largely used to investigate the mucosal host response, has been established in a Transwell system. When grown on permeable supports, Caco-2 cells polarise and form a monolayer mimicking the intestinal barrier. They have morphologically different apical and basolateral surfaces, with solid intercellular tight junctions, resembling enterocytes lining the small intestine.

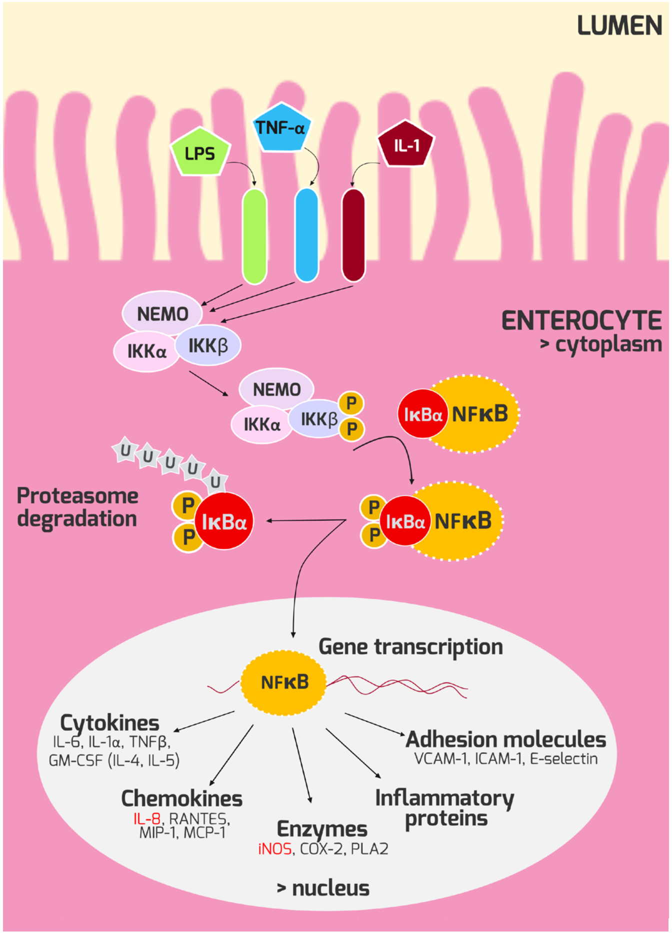

Figure 5. NF-κB pathway

The vegetative cells of the bacterial strains of interest, as well as the positive control, were incubated with the Caco-2 cells. The intestinal cells were then stimulated to mimic inflammatory response. The stimulation was carried out by exposing Caco-2 cells to interleukin 1 beta (IL-1 beta), the mycotoxin deoxynivalenol (DON) and flagellin (Fla). The level of inflammatory response was assessed for each treatment by measuring the production of proinflammatory cytokine, interleukin 8 (IL-8), by ELISA.

The results showed that IL-8 released by Caco-2 cells was increased by the three stimuli: IL1-β, DON and Fla (Figure 4). EGCG decreased IL-8 production in all stress conditions. When the inflammation was induced by IL-1 β, the highest anti-inflammatory effect was obtained by Bs 29784. In flagellin-mediated inflammation, it was observed that Bs A increased IL-8 production, whereas Bs B and Bs 29784 decreased it.

Interestingly, Bs A and Bs B actually potentiated the effect of DON on IL-8 production. Whereas, Bs 29784 still reduced

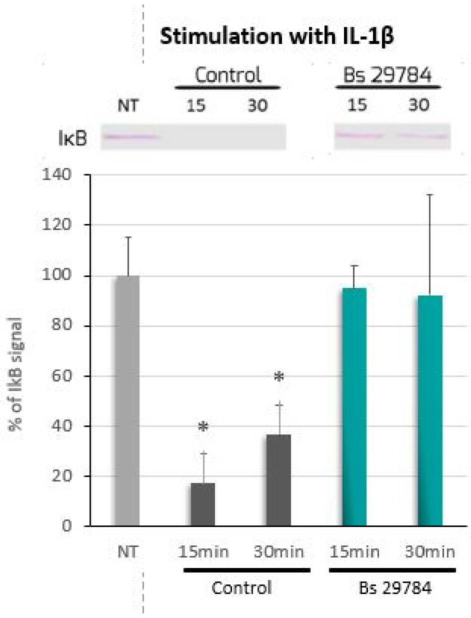

Figure 6: Effect of Bs 29784 on IκBα breakdown (IκBα cytolosic levels measurements

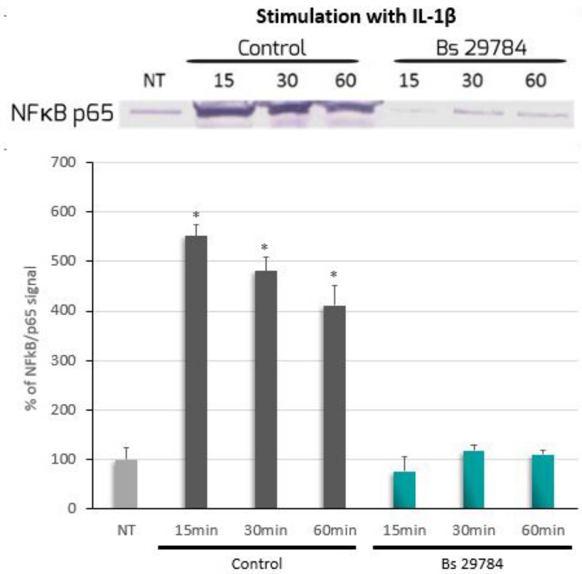

Figure 7: Effect of Bs 29784 on the nuclear translocation of NF-κB

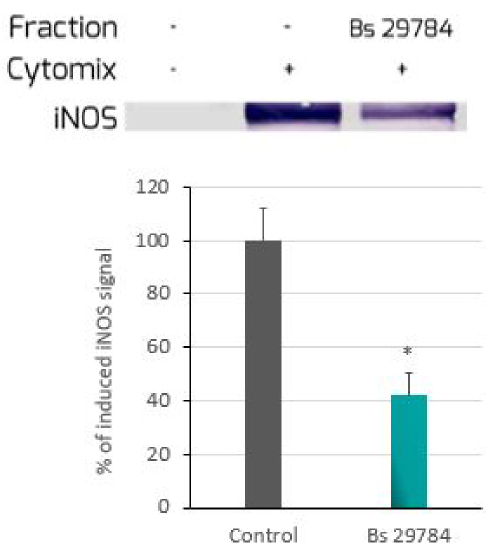

Figure 8: Effect of Bs 29784 on iNOS production

IL-8 production compared to DON-treated control. Together, these results further illustrate the differences between *Bacillus* strains. Since Bs 29784 showed a significant decrease in IL-8 release in all inflammatory conditions, this strain was subsequently investigated in more detail.

Modulation of the Inflammatory Response by *Bacillus subtilis* 29784

To better understand how Bs 29784 can exert its immunomodulatory properties, the NF-κB signalling pathway was investigated first.

NF-κB is well known to play a central role in regulating gene expression in a wide variety of cellular responses, including immune response and inflammation (Figure 5). The process works by firstly recognising various pro-inflammatory signals, from pathogens and toxins, by specific cellular receptors. Once linked to these receptors, the signal pathway is activated leading to the nuclear translocation of NF-κB and finally, to the action of gene expression. Among them, there are those coding for IL-8 and inducible nitric oxide synthase (iNOS). However, to be able to translocate into the nucleus, IκB, the inhibitor of NF-κB must first be degraded in the cytosol.

In the study, *Bacillus subtilis* 29784 was able to inhibit the degradation of IκB, in IL-1 stimulated conditions (Figure 6). This consequently led to the inhibition of NF-κB translocation into the nucleus (Figure 7), explaining the reduction in IL-8 production previously demonstrated; but also, the reduction of iNOS induction by pro-inflammatory cytokines (Figure 8). iNOS can produce large amounts of NO (nitric oxide), which helps destroy pathogens, but may be harmful if uncontrolled. Therefore, iNOS expression should not be completely inhibited but tightly regulated, as Bs 29784 seems to do, to prevent excessive inflammation.

***Bacillus subtilis* 29784 can Prevent the Disruption of the Intestinal Barrier**

Preserving the integrity of the intestinal barrier is critical for the performance and health of animals. As well as ensuring nutrient absorption, the intestinal barrier plays an important role in protecting the immune system. The integrity of the intestinal barrier is ensured by the epithelial tight junctions. They are multi-protein complexes that form a selectively permeable seal between adjacent epithelial cells and demarcate the boundary between apical and basolateral membrane domain.

The direct effect of Bs 29784 on intestinal barrier function has been assessed in a study in which the Trans-epithelial Electrical Resistance (TEER) was measured. TEER is a well-known *in vitro* method for evaluating the integrity of monolayer epithelial cells by measuring the voltage between the apical and the basolateral side. The higher the TEER is, the better the integrity is. *Bacillus subtilis* 29784 was shown to improve the TEER, thereby preventing the disruption of the intestinal barrier, under both stressed and standard conditions.

Using a Western blot assay, Bs 29784 was also shown to increase the expression of tight junction proteins; ZO-1, claudin-1 and occludin. This explains the improvement in TEER, which leads to the strengthening of the intestinal barrier.

Implications

Data from these assays showed that Bacillus-based probiotics may indeed improve digestive health. The strain *Bacillus subtilis* 29784 (Alterion®) is able to strengthen the intestinal barrier and control inflammatory responses, properties which are strain-dependent. It exerts its immunomodulatory properties by inhibiting IkB degradation, thus preventing NF-κB translocation and, by doing this, the expression of pro-inflammatory cytokines such as IL-8 and iNOS.

By controlling a pro-inflammatory response, this probiotic strain would allow poultry to reach their full genetic potential in terms of growth. Inflammatory responses use a significant amount of energy, which would otherwise be used for growth. Therefore, by reducing these responses, energy is saved, and growth optimised. As the use of AGPs is reduced, due to concerns over resistance, alternative solutions need to be proposed. If these are to have the same effect on production efficiency, alternatives need to positively influence each of the gut health pillars, including anti-inflammatory properties.

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Lamya Rhayat joined Adisseo seven years ago as a Digestive Health Expert. She has been involved since the beginning of the development of Alterion®, consistent probiotic solution. The project involved screening and selecting the strain – going further in understanding its mode of action through *in vitro* and *in vivo* studies. Lamya has a PhD in microbiology and a master's degree in immunology.

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