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**Capability of yeast derivatives to adhere
enteropathogenic bacteria and to modulate cells of
the innate immune system**

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Abstract

Yeast derivatives including yeast cell wall components are promising alternatives to antibiotics with respect to the promotion of health and performance in livestock, based on their capacity to bind enteropathogenic bacteria and to beneficially modulate the immune system. However, these mode(s) of action both *in vitro* and *in vivo* are still not well understood. Furthermore, standardization and reproducibility of *in vitro* techniques (microbiology, cell culture assays) are critical features for the application of yeast derivatives as well as for the proof of effectiveness.

Yeast cell wall products are suggested as anti-adhesive agents, and are thus proposed to prevent attachment of certain intestinal bacteria by providing alternative adhesion sites to enterobacteria, which contain mannose specific type-I fimbriae such as *E. coli* or *Salmonella* spp. and which is well documented. Various *in vitro* assay techniques have become of paramount importance for biotechnological research since they allow for determination and quantification of potential mode(s) of action. However, *in vitro* assays may be criticized by product end users as not accurately reflecting *in vivo* responses. Pro and cons of different assays and their bias will be discussed specifically regarding yeast cell wall components and adhesion of enteropathogenic bacteria. Immunomodulation is a therapeutic approach intervening in auto regulating processes of the defense system. Yeast derivatives such as beta-glucans are proposed to interact with cells of the innate immune system (e.g. cytokine) by receptor recognition. Controversial data in literature and mode(s) of action are reviewed and discussed here.

Key words: yeast derivatives, adhesion, enteropathogenic bacteria, immunomodulation, *in vitro* assays, cytokines

Chapter 1

Capability of yeast derivatives to adhere enteropathogenic bacteria and to modulate cells of the innate immune system

In preparation to submit to a peer reviewed Journal (Review article)

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Introduction

Since the second half of the 20th century the application of yeasts and nutritional yeast, and in general the concept of functional feed and functional food, became a matter of active discussion in many parts of the world, as an issue with increasing importance for industry and science. The world population is increasing, especially in the developing countries, and the challenge for the food and feed chain to provide wholesome and affordable food is getting increasingly demanding. The European Union ban of antibiotic growth promoters in 2006 together with a growing interest by the consumers in many industrialized countries all over the world in healthy and antibiotic-free food require innovative solutions for the world food supply. The World Health Organization is urging meat-producing countries around the world to use sustainable alternative methods of controlling infectious diseases (Kogut, 2000). The fact that animals can carry pathogenic bacteria that represent a potential risk for humans, sometimes even without causing symptoms in the animal itself is a serious public health issue. In addition, animals with gastrointestinal problems can cause considerable economic losses in livestock farming (Becker, 2005). Antibiotics have been used intensively for decades in livestock production as growth promoters to control intestinal pathogens, to reduce mortality and to improve animal health and performance. The ban on feeding growth-promoting levels of antibiotics (AGP) in a growing number of countries worldwide has led to greater interest in alternatives to AGPs, ideally while maintaining equivalent animal health and performance. One of the most important reasons given for banning prolonged use of AGPs is the potential for pathogenic bacteria to develop resistance to those antibiotics. Consequently, research on these alternatives to AGPs has been intensified, and beneficial effects on the immune system and on gut health have been suggested and discussed for some of these (Applegate *et al.*, 2010; Kogan and Kocher, 2007b; Sonnenburg *et al.*, 2004). Natural

alternatives such as yeast products, prebiotics, probiotics, acidifiers, and essential oils are currently in the focus of interest, and thus are increasingly being investigated.

1. Application of yeast as health promoter's

Yeasts are probably one of the earliest domesticated organisms. People have used yeasts for fermentation and baking throughout history. The most important and best known strain in food science undoubtedly is *Saccharomyces cerevisiae*. Yeast extract has importance as a flavor enhancer due to its high content of glutamic acid as well as ribonucleotides such as inosine monophosphate (IMP) and guanosine monophosphate (GMP). Yeast powder or yeast drinks are proposed as health-improving food supplements because of their high content in vitamins, mainly B vitamins, protein and minerals. Various probiotic supplements use *Saccharomyces boulardii* to improve gut health. Furthermore *S. boulardii* has been shown to reduce acute diarrhea in children, to prevent infection by *Clostridium difficile* and to reduce the incidence of antibiotic-, traveler's- and HIV/AIDS-associated diarrhea (Fuller, 1989; Marteau *et al.*, 2001; Saarela *et al.*, 2002).

In animal nutrition (livestock) live yeast and yeast products such as yeast cell walls have been discussed as health-promoting feed additives and as preventive antibiotic replacements for the past decades. Yeast products have been fed to animals for more than hundreds of years, and include yeast-fermented mash directly produced on the farm, yeast by-products from breweries and distilleries, or yeast products commercially produced for animal feeding. However, their *in vitro* and *in vivo* mode of action with respect to improving animal performance is still not completely understood. Live yeasts have been described as being capable of protecting the intestinal mucosa against invading microorganisms by being antagonistic to undesirable microorganisms (barrier effect), and by contributing to the stimulation and maturation of the immune response of host animals (Fuller, 1989). Yeast cell

wall components (e.g. mannan-oligosaccharides) reportedly prevent colonization of pathogens in the intestinal tract both by binding of those pathogenic bacteria which have mannose-specific type-I fimbriae as well as through their prebiotic activity, i.e., they are selectively fermented in the intestine and thus they allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota, resulting in an increase in the number of beneficial bacteria such as lactobacilli and bifidobacteria (Firon *et al.*, 1984; Gibson and Wang, 1994; Ofek and Beachey, 1978; Shoaf-Sweeney and Hutkins, 2008; Zopf and Roth, 1996). Beta-glucan, one of the constituents of the yeast cell wall, has been shown to stimulate the immune response, for example by increasing the numbers of activated phagocytic cells such as macrophages. Autolysed yeast has been proposed to possess dual beneficial effects of both immunomodulation and pathogen control/defense (Kogan and Kocher, 2007a). Finally, selenium yeast presents an excellent source of organic selenium such as selenomethionine and selenocysteine which is important for humoral and cellular immunity (Elmadfa and Leitzmann, 1998).

2. Yeast and control of pathogenic bacteria

2.1. Mode(s) of action, anti-adhesion approach

There is a significant need by the food and feed industry, as well as by human and veterinary medicine, to provide natural measures of defense against infectious diseases such as those caused by enterobacteria, e.g. *Salmonella* spp. and *E. coli*. Protecting human and animal health against diseases and infections is of paramount importance. According to EFSA's Second Community Summary Report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks in the European Union, 131,468 confirmed cases of human salmonellosis were reported in 2008. In foodstuffs, *Salmonella* was most

often detected in fresh broiler, turkey and pig meat. The most commonly reported serovars in humans and animals were *S. Enteritidis* and *S. Typhimurium*. In 2008, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union with 190,566 confirmed cases. Live poultry, pigs and cattle were the most common sources of *Campylobacter*. According to Much *et al.* (2007), 99% of the food borne outbreaks in Austria were accounted by *Salmonella* spp. and *Campylobacter* spp. in 2005.

Yeast derivatives such as various yeast cell wall products and recently also yeast autolysate products are sold worldwide as feed supplements with health claims of binding enteropathogenic bacteria such as *E. coli* and *Salmonella* spp.. “Not all yeast products are the same”, and hence there is enormous confusion throughout the feed industry concerning the quality of yeast products. An important initial event in bacterial pathogenesis is the adherence of bacteria via their surface lectins to host intestinal cells. Bacteria must first bind to the epithelial cells of these tissues to colonize the intestinal mucosal surfaces. Fimbriae, also called pili, are hair-like, proteinaceous appendages on the surface of bacteria. Fimbriae provide the bacteria with adhesive properties and are involved in the specific adherence of bacterial cells to host tissue; however in this context it has also to be noted that bacterial adhesion can also occur without fimbriae, namely by afimbrial adhesins which are located at the cell surface. The adhesive properties of fimbriae have been investigated in detail studying and elucidating criteria for bacterial pathogenicity and virulence (Vimal *et al.*, 2000; Zopf and Roth, 1996). Adhesion is based on binding of oligosaccharide components of host mucosa via certain lectins. Mannose-specific (type 1) fimbriae are among the most commonly found lectins in enterobacteria such as *E. coli* or *Salmonella* spp. (Firon *et al.*, 1984; Ofek and Beachey, 1978). Thus, infections are initiated only after the microorganism has first adhered to the host cell surface. If adherence can be inhibited then the subsequent

infection can also be inhibited. This approach forms the basis of anti-adherence strategies. The most studied of these anti-adherence approaches involve receptor-analogs, which include oligosaccharides (Shoaf-Sweeney and Hutkins, 2008). The use of agents that interfere with the ability of bacteria to adhere to tissues of the host provides an attractive approach to fight against bacterial infections (Ofek *et al.*, 2003b). Yeast cell wall products are suggested as anti-adhesive agents and thus proposed to prevent intestinal bacterial attachment by providing alternative adhesion sites to enterobacteria which contain mannose-specific type I fimbriae (Ofek *et al.*, 1977). According to literature mannan-oligosaccharides (MOS) are those components in the yeast cell wall that are responsible for binding of pathogens (Sharon and Ofek, 2000; Snellings *et al.*, 1997). However, it has been questioned recently, whether the total amount of mannose in the yeast cell wall is responsible for immobilizing enteric pathogens. A plethora of literature describes various adherence mechanisms and proposed mode(s) of action of yeast derivatives (Mourao *et al.*, 2006; Oyofe *et al.* 1989; Sharon and Ofek, 2000; Shoaf-Sweeney and Hutkins, 2008; Snellings *et al.*, 1997; Spring *et al.*, 2000; Terre *et al.*, 2007; Yang *et al.*, 2008). Shoaf-Sweeney and Hutkins (2008) propose that the adhesion affinity is defined by the valency of protein-oligosaccharide interactions. A single molecule thus has only low affinity, while the creation of individual protein subunits with numerous oligosaccharide-binding sites assembled to a filamentous structure leads to increased target avidity (Shoaf-Sweeney and Hutkins, 2008).

The three-dimensional structure of mannose chains within the yeast cell wall is a critical feature to be taken into consideration; Ganner *et al.* (2008) describes that this has significant influence on binding behavior of different yeast cell wall products. The three-dimensional structure is therefore presumed to be of importance for pathogen immobilization rather than the total amount of mannan. Indeed, quantitative, rather than qualitative assays are essential to evaluate the pathogen-binding capacity of cell walls from different yeast sources and

strains (Ganner *et al.*, 2008). Ofek *et al.* (2003a) describes that - apart from adhesion-receptor interaction - hydrophobic and other non-specific interactions under different shear-forces might be involved in the adhesion process as well.

Becker and Galletti (2008) investigated various food and feed components for alternative adhesion of intestinal *E. coli* and *Salmonella*. Yeast mannan-oligosaccharides, pumpkin, sesame seed extract, palm kernel extract and konjac gum were among the strongest binding matrices for enteropathogens. Adhesion matrices differ tremendously, and this makes interpretation and speculation about binding factors substantial. Thus, indication is given that unspecific, unknown binding properties are involved in this process. Moreover, taking the diversity of natural products due to biological variation for processing into account, the binding capacity of fibrous food and feed components for different bacteria seems rather unpredictable (Becker and Galletti, 2008).

2.2. *In vitro* assay techniques: pros and cons

The search for efficacious products as antibiotic replacements in livestock production has relied heavily on *in vitro* screening assays as well as on studies conducted with rats. Various *in vivo* studies performed using piglets and broilers have highlighted the benefits of yeast cell wall on animal health and performance suggesting that blocking of the mannose receptors, which allow pathogens to adhere to the gut mucosa, may reduce or eliminate enteric bacterial infections (Mourao *et al.*, 2006; Oyofe *et al.*, 1989; Spring *et al.*, 2000; Terre *et al.*, 2007; Yang *et al.*, 2008). To justify and corroborate these *in vivo* studies, additional *in vitro* studies have clear importance for further research to explain the potential mode(s) of action.

However, *in vitro* assays may be criticized by product end users as not accurately reflecting *in vivo* responses. This sentiment may be true in some cases, but in others it may partially be a reflection of assay bias attributable to media selection, duration, or substrate concentration.

Nevertheless, *in vitro* assay techniques have become of paramount importance for biotechnological and pharmaceutical research since they allow for determination of potential mode(s) of action, allow for higher throughput screening of product candidates, and are not influenced by environmental factors that may mask *in vivo* results (temperature, disease pressure, etc.) and pharmacokinetics. Linking of *in vitro* techniques to *in vivo* viability and efficacy has been both well used and abused in the literature. One case in point is the *in vitro* literature regarding yeast cell wall components which will be discussed subsequently (Applegate *et al.*, 2010).

As mentioned previously mannan-oligosaccharides (MOS) have been proposed to have a “weakening” effect on gram-negative pathogenic bacteria such as *E. coli* and *Salmonella* spp.. It has been proposed that MOS are able to bind and move pathogens through the gut without colonization, thus lessening or preventing enteric disease(s). When performing *in vivo* studies, researchers have not measured (and, given the current methodology, cannot measure) direct binding with pathogens, but rather have indicated reduced numbers of selected bacteria in intestinal digesta and feces (Baurhoo *et al.*, 2007; Spring *et al.*, 2000). Worldwide, some animal feed industry personnel are claiming these health benefits, using *in vitro* methods to support this statement. One method of studying the interaction of MOS with bacteria has been via the agglutination method. This method mixes bacteria with a cell wall solution/suspension, followed by visual evaluation of binding (Mirelman *et al.*, 1980; Ofek *et al.*, 1977; Spring *et al.*, 2000). Critical issues with this procedure and its interpretation are the poor solubility of the MOS in aqueous dilutions as well as the qualitative nature of the assay itself. Solubility of the MOS solution is not usually stated despite of their high molecular weight, which typically results in fast sedimentation of the heavy cell wall fractions. Visual evaluation of binding is highly subjective because it is not possible to determine whether the bacteria are actually bound or whether they are merely in proximity to the yeast. Therefore,

the use of this assay as an adequate *in vitro* tool is unsatisfactory since typically it is neither well reproducible nor quantitative across a range of products. Another method used to study the interaction of yeast cell walls with bacteria has been via the sedimentation method. Newman *et al.* (2006) used this procedure, assuming that bacteria that are attached to the yeast cell wall will sediment; thus, the faster the sedimentation, the better the binding between bacterial cells and yeast cell walls. However, this procedure can also be criticized since all bacteria and yeasts (or yeast cell wall fractions) will actually sediment in aqueous solutions because of their polarity and molecular weight. A weak point for *in vitro* assays mentioned so far is indeed the lack of quantitative analytical methods to determine the total amount of bacteria bound by yeast cell walls. This may be overcome by a third method used to study the interaction of yeast cell walls with bacteria, which is based on a microtiter plate. In this procedure one assumes that the insolubility of the cell wall material given by its high molecular weight can be taken as an advantage since the cell wall material is used to coat the wells. The wells are allowed to incubate with the test bacteria, unbound bacteria are rinsed out, and the growth rate of bound bacteria is subsequently quantified. The accuracy and reproducibility of binding is given by automated evaluation of optical density (Becker *et al.*, 2007; Ganner *et al.*, 2010; Sokurenko *et al.*, 1995). Ganner *et al.* (2010) investigated furthermore concentration-dependent pathogen binding of yeast cell wall as well as quantitative binding differences between strains and serovars.

Standardization of experimental conditions is important and necessary to achieve reproducible results and quantitative measurement of bacterial adhesion to evaluate the binding capacity of yeast cell wall. Moreover, quantitative measurement of the bacterial adhesion is necessary to evaluate the binding capacity and the quality of yeast derivatives. Because of substantial variation between assays, their application should be scrutinized for product evaluation. Peer reviewers should be cognizant and diligent of this and ascertain that

in vitro screening tests described in scientific manuscripts are in fact validated for appropriateness and reproducibility, and that the relevance of results is carefully interpreted (Applegate *et al.*, 2010).

3. Yeast and Immunology

3.1. Innate and adaptive immune cells, immunomodulation

The principle responsibilities of the immune system are to recognize and to destroy a wide variety of pathogens while tolerating the tissues of the host organism. The innate immune system has evolved over the hundred million years and includes physical barriers such as skin and epithelia, as well as cells such as macrophages (phagocytosis), dendritic cells, neutrophils, basophils, eosinophils, mast cells and natural killer cells. An encounter with a pathogen leads efficiently to the prompt removal. Phagocytosis initiates the production of cytokines and plays a central role in protection against microbial infections. Cytokines are biologically active protein combinations, mostly glycoproteins that influence the activities of other immune cells and play a central role in controlling the host immune responses and the control of inflammatory responses. Furthermore, cytokines participate in the regulation of phagocyte activities and as such are used for immunomodulatory therapy (Gallova *et al.*, 2004).

The specific immune response can be classified into humoral and cellular immunity. Humoral immunity is affected by antibodies produced by plasma cells (mature B-lymphocytes), which bind specifically to antigenic epitopes on the surface of pathogenic organisms and with the aid of complement can kill these pathogens. There exist different classes of antibodies (IgA, IgG, IgM, IgE) with specific functions. Cellular immunity is mediated by T-lymphocytes. On exposure to an antigen or pathogen, T-lymphocytes proliferate and produce cytokines.

One of the primary functions of the immune system is to mediate protection against disease. Infectious diseases occur as a result of interaction between pathogenic microorganisms and the host. All infectious diseases begin at some surface of the host, whether internal or external. Some pathogens, such as *Salmonella*, *Shigella*, *Listeria*, may be capable of growing within the cells of the host, causing severe disruption of normal physiological processes resulting in acute diarrhea, nausea and vomiting (Pelczar *et al.*, 1986). Extracellular bacteria for instance, are eliminated and controlled by physical barriers, phagocytes, complement and antibodies. Intracellular bacteria, such as *Salmonella* spp., (*Shigella* spp., *Listeria* spp.), are controlled following the activation of T-lymphocytes and the secretion of cytokines such as IFN-gamma which activate monocytes /macrophages. Most successful host immune responses involve both humoral and cell-mediated responses, as well as their interaction with the non-specific immune system.

The immune/defense system is an autoregulating system. Immunomodulation is defined as shifting the immune response to pathogens or other exogenous triggers. In other words, immunomodulation is a therapeutic approach, in which we aim at intervening in autoregulating processes of the defense system. The underlying principle is that the immune system can be improved by an informed intervention. Implicit are the idea of mal-regulation and the “cost of energy” which result in loss of animal performance. Immunomodulation is described as being situation-dependent: improving disease resistance against some pathogens while impairing it against other pathogens with different pathogenicity mechanisms. Thus, when the type of immune response is clearly understood, an immunomodulator that is known to shift the immune response in the protective direction is good for that single pathogen; but not necessarily for others (Klasing, 2010). Immunomodulators alter activity of immune function through the dynamic regulation of informational molecules (e.g. cytokines) (Gallova *et al.*, 2004).

3.2. Yeast derivatives as immunomodulators

Yeast derived polysaccharides such as beta-glucans have been considered as potential growth and health promoters by beneficially modifying the immune system. Numerous *in vitro*, *ex vivo* and *in vivo* studies, summarized by Volman *et al.* (2008) and Kogan and Kocher (2007b) describe the immunomodulating effect of beta-glucans such as enhanced functional status of macrophages, increased cytokine production and oxidative burst (NO) by macrophages, neutrophils and dendritic cells, chemokine production by (alveolar) epithelial cells, increased leukocyte activity including increased survival against pathogen infections. Furthermore also anti-tumor as well as antioxidant activities are described (Kogan and Kocher, 2007b).

Li *et al.* (2005) reported that dietary beta-glucan attenuated the increase of pro-inflammatory cytokines (IL-6, TNF-alpha) whereas the increase of IL-10 was potentiated in an *in vivo* study with LPS-challenged weaning piglets. However, in another study Li *et al.* (2006) observed a trend towards elevated pro-inflammatory (IL-6 and TNF-alpha) plasma cytokines in LPS challenged piglets. Mao *et al.* (2005) described improved lymphocyte proliferation of LPS challenged pigs fed with beta-glucan. Pro-inflammatory cytokines are among the first cytokines produced in response to pathogenic bacteria (Miettinen *et al.*, 1998). IL-18, also known as IFN-gamma activating factor, is an important regulator of innate and acquired immune responses, and has also been described to enhance host defense (Dinarello, 1999; Dinarello, 2000). IL-18 produced by activated macrophage-like cells acts synergistically with IL-12 by enhancing IFN-gamma gene expression in T-cells (Miettinen *et al.*, 1998). Previously it has been demonstrated that both IL-12 and IL-18 promote inflammatory response; however there is a post-weaning decrease in gene expression of piglets reflecting either an adaptive immune response to the weaning transition or a consequence of the maturation of the intestinal immune system (Pie *et al.*, 2004).

The mannan-oligosaccharide fraction of the yeast cell wall can modulate, in part, the immune system by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Ferket *et al.*, 2002).

Heterophils are leukocytes which help the body to fight against infectious diseases and foreign substances. Heterophils belong to the innate immune system; have the ability - such as macrophages - to phagocyte bacteria and parasites to protect the animal against primary infections. The heterophil is a highly phagocytic, granulated cell capable of antimicrobial activity and is analogous, although not identical, to the mammalian neutrophil (Harmon, 1998; Huff *et al.*, 2007). Huff *et al.* (2007) reported an increase in heterophil (percentage of leucocytes) in whole blood in a study, in which turkeys were fed yeast extract and challenged with *E. coli*. While activation of heterophils is an important component of the innate immune response, heterophils are also responsible for tissue damage by accumulation in inflamed tissue and forming heterophil granulomas which are morphological similar to inflammatory lesions in reptiles (Harmon, 1998). In mammals, stress and infection have been shown to increase the rate of neutrophil production by 10-fold (Smith, 1994).

Cells of the innate immunity such as macrophages, phagocytes and natural killer cells have surface beta-glucan receptors, which specifically recognize and bind the beta-1,3 -glucan linkage of the beta-glucan molecule (Brown and Gordon, 2001; Engstad and Robertsen, 1994). The most active immune stimulators appear to be branched chain 1,3-beta-D-glucans, sometimes referred to as 1,3- and 1,6-beta-D-glucans, present in the yeast cell wall. These branched chain glucans have the capacity to activate the innate immune system, thereby enhancing the defense barriers and providing protection against a variety of infections (Raa, 1996; Stuyven *et al.*, 2009). Several different receptors on phagocytic cells that recognize beta-glucans have been identified, such as CR3 (Xia *et al.*, 1999) and Dectin-1 (Brown and Gordon, 2001). Apart from the mentioned receptor recognition on macrophages, dendritic

cells and eosinophils surface and as a result the activation of the immune cells, underlying mechanisms such as digestion by epithelial macrophages after oral beta-glucan administration are discussed as immunomodulating triggers (Volman *et al.*, 2008). Stimulation of the intestinal immune system by modulation of the mucosal immune response by cells of Peyer's patches as well as intestinal intraepithelial lymphocytes (increased cell numbers) and enterocytes (apoptotic numbers) are reviewed and discussed by different authors (Tsukada *et al.*, 2003; Volman *et al.*, 2008; Applegate *et al.*, 2009). However, the exact specificities of different receptors and underlying mechanisms have not been fully elucidated.

Additionally, yeast cell wall components, such as mannan-oligosaccharides and beta-glucans have been purported to have structural intestinal health benefits. Baurhoo *et al.* (2007), Baurhoo *et al.* (2009) and Solis De Los Santos *et al.* (2007) investigated mannan-oligosaccharide preparations in chickens and observed improved gut morphology including longer villi, shorter crypts and enhanced goblet cell numbers in the villus. Beyond the surface area improvement, goblet cell numbers are protective because of their production of intestinal mucus. Mucus has several functions such as lubricating intestinal surfaces, trapping and neutralizing bacteria, detoxifying heavy metals by binding, interacting with the intestinal immune system, acting as a diffuse barrier for nutrients and macromolecules thus protecting the underlying epithelial cells (Forstner and Forstner, 1994). Another important function of mucin is that the structure of the mucus provides several potential attachment sites and colonization niches for commensal and pathogenic bacteria (Sonnenburg *et al.*, 2004).

The significance of immunomodulatory activities of fungal beta-1,3-glucans is described in literature being dependent on the structure, such as solubility in water, molecular weight, degree of branching, and conformation. Nitric oxygen production by macrophages has been shown to be conformation dependent, while e.g. interferon gamma was conformation

independent (Yadomae, 2000). Okazaki *et al.* (1995) demonstrated and discussed that the branching ratio and molecular weight of beta-1,3-D-glucans are important factors for TNF-alpha production from macrophages. TNF-alpha release by macrophages is induced only by beta-glucans with high molecular weights and lower branching ratios, and the mechanism for the recognition of beta-glucans is multiple and assumed to be divided into several parts involving various cellular functions (Okazaki *et al.*, 1995). However, Kulicke *et al.* (1997) observed in his study -focusing on the effect of glucans with different structures and different molecular mass on TNF-alpha production of human blood monocytes- no correlation between helical structure and immunological activity.

Although many research studies have described that 1, 3- beta-D-glucans elicit the production of cytokines and nitric oxide, the relationships between the structures of the glucans and their stimulatory activities are still controversial due to a lack of reproducible *in vitro* systems to evaluate the activities of 1,3-beta-D-glucan and due to the biological diversity (Kataoka *et al.*, 2002). Furthermore the characterization and chemical analytics of beta-glucan structure and branching is challenging and crucial.

Taken the above mentioned points into consideration it should be stated that the manufacturing processes and the quality control methods are very critical stages when yeast derivatives should be brought to the market as effective animal feed additives. Isolation methods of beta-glucans may influence strongly the quality, the branches as well as the structure of this complex molecule and as a result its biological activity.

Taken together, numerous *in vitro* and *in vivo* trials have substantiated given effects proving a strong positive influence of yeast derivatives (mainly beta-glucans) on the immune system of humans and animals. However, the mechanisms underlying the studied health beneficial actions remain largely unknown and future multidisciplinary research within this field is needed to illuminate observed positive effects.

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**Quantitative *in vitro* assay to evaluate the capability of
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Quantitative *in vitro* assay to evaluate the capability of yeast cell wall fractions from *Trichosporon mycotoxinivorans* to selectively bind gram negative pathogens

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ABSTRACT

Yeast cell wall fractions have been proposed to bind enteropathogenic bacteria. The aim of this study was to develop a quantitative assay by measuring the optical density as growth parameter of adhering bacteria. The exponential growth phase of adhering bacteria was determined by optical density reading and compared with the colony count (CFU/mL). A linear regression was compiled and the bacterial number bound to the yeast cell wall product could be determined. Further focus was the investigation of a yeast cell wall from strain *Trichosporon mycotoxinivorans* (MTV) for its ability to bind gram negative *Salmonella*, *E. coli* and *Campylobacter* strains and gram positive probiotic bacteria of the genera lactobacilli and bifidobacteria as well as gram positive *Clostridium perfringens* quantitatively. The gram negative probiotic strain *E. coli* Nissle 1917 was also investigated. Seven out of 10 *S. Typhimurium* and *S. Enteritidis* strains adhered to the cell wall product with an amount between 10^3 and 10^4 CFU/10 µg. Four out of 7 *E. coli* strains showed an average binding capability (10^2 CFU/10 µg) whereas 4×10^3 *E. coli* P4 cells bound per 10 µg yeast cell wall. *E. coli* 0149 K91, *E. coli* 0147 K89, *C. jejuni* and *C. perfringens* as well as the genera lactobacilli and bifidobacteria did not bind to the yeast cell wall. *E. coli* Nissle 1917 was bound with 2×10^2 CFU/10 µg. These results demonstrate that cell wall from MTV can be used to differentially bind *E. coli* spp. and *Salmonella* spp. up to 8×10^4 CFU/10 µg. Thus certain yeast cell walls may prevent enteric infections caused by selective bacteria. This methodical approach would be an accurate tool in the feed industry for quality control of yeast cell wall products.

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1. Introduction

There is significant need by the food and feed industry, as well as by human and veterinary medicine, to provide natural measures to defend against infectious diseases such as those caused by enterobacteria, e.g. *Salmonella* spp. and *E. coli* spp.

Protecting human and animal health against diseases and infections is of paramount importance. According to EFSA's Second Community Summary Report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and food borne outbreaks in the European Union in 2008, 131,468 confirmed cases of human salmonellosis were reported. In foodstuffs, *Salmonella* was most often detected in fresh broiler, turkey and pig meat. The most commonly reported serovars in humans and animals are *S. Enteritidis* and *S. Typhimurium*. In 2008, campylobacteriosis continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union with 190,566 confirmed cases. *Campylobacter* sources were most commonly live poultry, pigs and cattle. According

to Much et al. (2007) in 2005, 99% of the food borne outbreaks in Austria were accounted by *Salmonella* spp. and *Campylobacter* spp.

Various *in vivo* studies with piglets and broilers have highlighted the benefits of yeast cell wall on animal health and performance suggesting that blocking of the mannose receptors, which allow pathogens to adhere to the gut wall, may reduce or eliminate enteric bacterial infections (Mourao et al., 2006; Oyofo et al., 1989; Spring et al., 2000; Terre et al., 2007; Yang et al., 2008). To justify and underline those *in vivo* studies, *in vitro* studies to explain the potential mode of action have clear importance for further research. An important initial event in bacterial pathogenesis is the adherence of bacteria via their surface lectins to host intestinal cells. Bacteria must first bind to the epithelial cells of these tissues to colonize the intestinal mucosal surfaces. Fimbriae, also called pili, are hair-like, proteinaceous appendages on the surface of bacteria. Fimbriae provide the bacteria with adhesive properties and are involved in the specific adherence of bacterial cells to host tissue; however in this context it has also to be noted that bacterial adhesion can also occur without fimbriae, namely by afimbrial adhesins which are located at the cell surface. The adhesive properties of fimbriae have been investigated in detail seeking criteria for bacterial pathogenicity and virulence (Vimal et al., 2000; Zopf and Roth, 1996). The adhesion is caused by binding of oligosaccharide components of host mucosa by means of lectins. Mannose-specific (type 1) fimbriae are among the most commonly

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found lectins in enterobacteria such as *E. coli* spp. or *Salmonella* spp. (Firon et al., 1984; Ofek and Beachey, 1978). Certain yeast cell wall products are proposed to protect the gastrointestinal tract through alternative adhesion sites to enteropathogenic bacteria. Mannan-oligosaccharides (MOS) are the substances in the yeast cell wall which are considered responsible for binding of pathogens (Sharon and Ofek, 2000; Snellings et al., 1997). According to literature (Klis et al., 2006) cell wall of *Saccharomyces cerevisiae* contains approximately 35–55% beta-glucan, 30–50% mannoprotein and 1.5–6% chitin. The mannoproteins, that form the outer cell wall layer, are highly glycosylated with a carbohydrate fraction that often amounts to over 90%. The cell wall glucans are composed of a 1,3-glycosidic backbone and 1,6-glycosidic intermediate chains, which together form a three-dimensional structure (Jaehrig et al., 2008; Klis et al., 2002). Cell wall of *Trichosporon mycotoxinivorans* has been described by Molnar et al. (2004) to compose approximately 59% glucose and 16% mannose.

Some qualitative test procedures such as the agglutination method combined with visual examination are described in literature investigating the binding capacity of yeast cell wall products (Eshdat et al., 1981; Mirelman et al., 1980; Oyofe et al., 1989; Peng et al., 2001; Spring et al., 2000). Sokurenko et al. (1995) and Becker et al. (2007) described the growth of adherent microorganisms by measuring the optical density and developed a quantitative approach by correlating optical density and colony count (CFU/mL). However, no further focus on yeast cell wall fractions and concentration-dependent pathogen binding of yeast cell walls has been undertaken. Furthermore, quantitative binding differences between strains and serovars have not been investigated. Indeed there is no standardized test system available which describes the yeast cell wall-pathogen adherence sufficiently. Standardization of experimental conditions is important and necessary to achieve reproducible results and quantitative measurement of bacterial adhesion to evaluate the binding capacity of yeast cell wall. Therefore in the present study, a quantitative *in vitro* microplate assay was developed by measuring the optical density as growth parameter of adhering bacteria based on the method of Becker et al. (2007). In addition we monitored the ability of yeast cell wall products from *T. mycotoxinivorans* to bind *Salmonella*, *E. coli*, *Campylobacter* and *Clostridium* strains as well as probiotic bacteria of the genera *Lactobacilli* and *Bifidobacteria* and the gram negative probiotic *E. coli* strain Nissle 1917 quantitatively.

2. Material and methods

2.1. Bacteria

Escherichia coli strains (08 K88 F4; 0108 K; 0139 K82; 0149 K91; 0108 K—; 0141 K85ac; 0138 K81; 0147 K89) were kindly provided by Dr. Hofer of the Austrian Agency for Health and Food Safety (AGES), Vienna, Austria following isolation from the gastrointestinal tract of piglets with diarrhea infection.

E. coli strain Nissle 1917 was obtained as a commercial probiotic source (Mutaflor, Ardeypharm GmbH, Germany).

All *E. coli* strains were transferred to Tryptic Soy Broth (TSB CM029, Oxoid, England) and subcultured in the same medium. Cells were incubated overnight at 37 °C under aerobic conditions, transferred to fresh medium and harvested in the log phase. Serially diluted cultures were enumerated on Tryptic Soy Agar (CM0131, Oxoid).

Salmonella enterica subsp. *enterica* (DSMZ 5569, 10506, 50912, 11320, 9274) were obtained from DSMZ, Braunschweig, Germany. *Salmonella enterica* subsp. *enterica* serovar Typhimurium S3 was obtained from AGES, Graz, Austria. *Salmonella enterica* subsp. *enterica* serovar Typhimurium Bio 60, *Salmonella enterica* subsp. *enterica* serovar Enteritidis Bio 59 and *Salmonella enterica* subsp. *enterica* serovar Choleraesuis Bio 63 were obtained from USDA, ARS.

All *Salmonella* strains were cultured under aerobic conditions at 37 °C to log phase growth for 18 h in Tryptic Soy Broth (TSB CM029,

Oxoid, England) prior to harvesting. Serial dilutions of freshly prepared cultures were plated on Tryptic Soy Agar (CM0131, Oxoid) and incubated for 24 h under aerobic conditions, prior to enumeration.

Clostridium perfringens 0307021 (Type A, CCUG 47895) was obtained from CCUG, Göteborg, Sweden. *C. perfringens* was cultured under anaerobic conditions to log phase growth for 48 h in Reinforced Clostridial Medium (CM0149, Oxoid) prior to harvesting. Serial dilutions of freshly prepared cultures were plated onto Reinforced Clostridial Agar (CM0151, Oxoid) and cultured for 48 h at 37 °C under anaerobic (CO₂ aeration) conditions, prior to enumeration.

Campylobacter jejuni SS LIO 103-920305 (CCUG 25903) was obtained from CCUG, Göteborg, Sweden. *C. jejuni* was cultured under microaerophilic conditions (CampyGen, Oxoid) to log phase growth for 72 h in Brain Heart Infusion (BHI, CM0225, Oxoid) prior to harvesting. Serial dilutions of freshly prepared cultures were plated onto Campylobacter Blood-Free selective agar base (CM739, Oxoid) and cultured for 48 h at 42 °C under microaerophilic conditions (CampyGen, Oxoid) prior to enumeration.

Lactobacillus salivarius (DSMZ 16351), *Enterococcus faecium* (DSMZ 19764) and *Lactobacillus reuteri* (DSMZ 21288) were isolated from piglet or poultry gastrointestinal tract (Klose et al., 2006). *Lactobacilli* and *Enterococcus faecium* were cultured under anaerobic conditions (CO₂ aeration) to log phase growth between 24 and 48 h in MRS broth (CM0359, Oxoid) prior to harvesting. Serial dilutions of freshly prepared cultures were plated onto MRS agar (CM0361, Oxoid) and cultured between 24 and 48 h at 37 °C under anaerobic conditions (CO₂ aeration) prior to enumeration.

Bifidobacterium animalis (DSMZ 16284) and *Bifidobacterium thermophilum* (DSMZ 19756) were isolated from poultry gut (Klose et al., 2006). The bifidobacteria were cultured under anaerobic conditions (CO₂ aeration) to log phase growth between 24 and 48 h in bifido-media (0.1% peptone, 0.1% yeast extract, 0.1% glucose, 0.03% KH₂PO₄, 0.01% Tween 80, 0.005% L-cysteine-HCL) prior to harvesting. Serial dilutions of freshly prepared cultures were plated onto the respective bifido-agar and cultured between 24 and 48 h at 37 °C under anaerobic conditions (CO₂ aeration) prior to enumeration.

All bacterial suspensions were adjusted to OD₆₉₀ 0.1 and a 1:10 dilution was taken for the experiments.

Calibration curves (linear regression) were produced for the bacterial genera *E. coli* and *Salmonella* independently, by plotting the beginning of the exponential phase of the adhering bacteria (= time in hours when broth medium reaches an optical density of 0.1 at 690 nm due to bacterial growth) versus agar counts of freshly prepared serially diluted cultures (in order to calculate viable numbers of bacterial cells bound to the yeast product).

2.2. Yeast derivative

The yeast derivative contains cell wall fragments derived from the strain *T. mycotoxinivorans* (MTV). Cell wall fragments were obtained by incubating MTV in 1000 ml shaking flask containing reverse osmosis water with 1% yeast extract and 3% saccharose for 40 h at 35 °C. Subsequently yeast cells were autolysed by incubation at pH 5, at 50 °C for 24 h and centrifuged (17,700×g, 10 min) to separate cell wall fragments from the supernatant. Finally the cell wall fragments were freeze dried (24 h, −30 °C/+30 °C, 0.001 mbar) (Epsilon 2-90, Christ, Osterode, Germany).

Autolysis was adapted according to Liu et al. (2008); the degree of cell rupture was monitored by increasing amount of protein- and carbohydrate content as well as dry matter in the supernatant. Peak protein and carbohydrate concentration as well as dry matter were determined after 24 h. Additionally the progress of autolysis was observed under the microscope (1000 times magnification) and compared to the original culture.

2.3. Microplate-binding assay

2.3.1. Principle

The quantitative adhesion of *E. coli* spp. or *Salmonella* spp. on yeast cell wall product was determined by measuring the optical density of the culture solutions, which indicates the growth of the adhering bacteria. The growth rate depended on the number of adhering bacteria—with higher numbers bound resulting in faster growth and thus the bacteria entering earlier into the exponential phase. The assay procedure is described below in detail for *E. coli* F4. *Salmonella*, *C. perfringens*, *C. jejuni*, lactobacilli and bifidobacteria require their respective nutrient media and agar as well as their respective incubation times as described in the bacteria section (Section 2.1).

2.3.2. Assay

Yeast cell wall product of different concentrations (0.01% and 0.001%) was suspended in phosphate buffered saline (PBS) and homogenized for 4 min in an ultrasonic bath (Labsonic U, B.Braun, USA). One hundred microliter aliquots were pipetted into each well of a 96-well flat-bottom high binding ELISA plate (IWAKI, Japan) and incubated for 16–18 h at 4 °C. The plate was washed three times with PBS-Tween20 using a microplate washer (Tecan 5082 M8/4R Columbus Plus). Subsequently, the plate was blocked for 1 h with 300 µL 1% Bovine Serum Albumin (BSA), then again washed three times. *E. coli* F4 was cultured aerobically overnight at 37 °C in Tryptic Soy Broth (TSB CM0129, Oxoid, England). On the second day 100 µL of this bacterial suspension was added to 10 mL fresh TSB and incubated for 2–3 h at 37 °C. The suspension was adjusted to OD 0.1 and aliquots of 100 µL of the respective 1:10 dilutions were added to the wells of the microplate. The bacteria were allowed to adhere to the yeast cell wall product for 60 min at 37 °C. Afterwards, the wells were washed 6 times with PBS-Tween20 to remove non-adherent bacteria. The wells were filled with 200 µL of TSB (or the media of the respective strain) and coated with one drop paraffin-oil to avoid dehydration. Blank, growth controls, negative control and blocking control were also assessed. The microplate was placed in a microplate reader (Tecan Genius, Spectra Flour plus, Austria), incubated for 18 h at 37 °C and measured at an OD of 690 nm. Data were recorded every 15 min.

Serial dilutions of *E. coli* (or the respective bacterial strain) were prepared and applied in parallel on the microplate and the agar plate in order to establish a linear regression between the number of counted bacteria on the agar plate (CFU/mL) and the time in hours when the adhering bacterial solution reached the exponential phase (optical density of 0.1 at 690 nm). Independent calibration curves were created for the genera *E. coli* and *Salmonella*. Subsequently, the number of bacteria bound to the yeast product was determined using linear regression.

The “binding control” consisted of BSA and test bacteria but without adding yeast cell wall product to determine the non-specific binding sites. The binding control had no growth until after 6 h. Finally the binding control, the bacterial number, which adhered to the non-specific binding sites of the well, was subtracted from the calculated data to determine the final number of specifically adhering bacteria. The blank consisted of the yeast cell wall product and BSA but without adding test bacteria and included all the assay steps to determine the bacterial number of the yeast cell wall product. The blank had no growth until after 10 h. The media control (TSB or MRS) had no growth until after 18 h.

The calibration is valid only for the respective genera and at an optical density of 0.1.

3. Results

The present results revalue the development of a standardized microbiological microplate based test assay and the verification of the binding properties to the cell wall of *T. mycotoxinivorans* of different

enteropathogenic *Salmonella* and *E. coli* strains, *C. perfringens*, *C. jejuni*, lactobacilli, bifidobacteria and the gram negative probiotic strain *E. coli* Nissle 1918.

The quantitative adhesion between bacteria and yeast cell wall was determined by measuring the growth of the adhering bacteria using the optical density of culture solutions. The growth rate depended on the number of adhering bacteria—with higher number bound resulting in faster growth and thus the bacteria enter earlier into the exponential phase in this experiment.

A linear regression (Fig. 1a,b) was compiled independently for the genera *E. coli* and *Salmonella* by plotting the CFU/mL against the detection time of serial dilutions (Fig. 2) of bacterial growth reaching an optical density of 0.1. Using the equation of the linear regression the unknown amount of bound bacteria (*E. coli* or *Salmonella*) was calculated according to the detection time. In a linear measuring range, the measuring window of the used photometer allowed detection of all cell densities between 10^1 and 10^8 CFU/mL (Fig. 2).

The quantitative adhesion numbers (CFU/10 µg) between bacteria and cell wall of MTV are listed in Table 1. *E. coli* strains had a binding capacity between 0 and 4×10^3 CFU/10 µg, with the highest binding rate shown by *E. coli* F4. In Fig. 3 delayed growth curves of *E. coli* F4 bound to different concentrations of yeast cell wall—between 10 µg and 0.01 µg—are shown. 10 µg of yeast cell wall binds 4×10^3 CFU, 1 µg of yeast cell wall 4×10^2 CFU, 0.1 µg of yeast cell wall 4×10^1 CFU and 0.01 µg of yeast cell wall 4 CFU. The ten *Salmonella* strains investigated showed a binding capability between 1×10^2 and 8×10^4 CFU per 10 µg of the cell wall preparation of MTV (Table 1). Fig. 4 shows the binding of *S. Typhimurium* to MTV cell wall; 10 µg yeast cell walls bind

Fig. 1. Linear regression for *E. coli* spp. (a) and *Salmonella* spp. (b); genus and threshold specific. Detection time: time in hours when bacterial growth reaches optical density of 0.1.

Fig. 2. Growth controls, dilution series of *E. coli* F4: Each dilution step was conducted in triplicates and ran in parallel to each experiment; kinetic measurements were recorded every 15 min over an 18 h period.

1×10^4 CFU, $1 \mu\text{g}$ yeast 1×10^3 CFU *C. perfringens*, *C. jejuni*, the lactobacilli and the bifidobacteria did evidently not show any binding in these tests as there was no growth between the bacteria and the yeast cell wall. In Fig. 5 the incubation of *L. salivarius* and cell wall of MTV, which resulted in no binding is demonstrated. *E. coli* Nissle 1917 shows similar binding behavior as the enteropathogenic *E. coli* strains with a quantity of 2×10^2 CFU/10 μg cell wall. Growth curves are shown in Fig. 6.

Each experiment with enteropathogenic or probiotic strains was done in triplicates and each experiment was repeated once. The coefficient of variation between intra assays was approximately 30%; and approximately 5% between the inter assays after optimization of the technique. Optimization techniques included accurate time management regarding strain incubation, strain density, yeast cell wall concentration, homogenization of cell wall aliquot, automated washing steps and sterile techniques.

4. Discussion

Yeast cell wall fractions have been proposed to bind enteropathogenic bacteria and therefore to have prophylactic properties in the gut for the control of selective pathogenic bacteria such as *E. coli* and *Salmonella*. A novel routine tool for quality control in the feed supplement industry has been developed within this study.

Standardized methods for studying the capability of yeast cell wall to bind pathogenic bacteria are scarce, even though they are sold as feed supplements in animal nutrition worldwide with claims to bind pathogenic bacteria. The adhesion properties of pathogens to mannose-containing yeast cell wall are described in the literature mainly with agglutination techniques (Mirelman et al., 1980; Peng et al., 2001; Spring et al., 2000). This method uses a suspension of yeast cell wall and a bacterial solution, which is observed under the microscope (1000 times magnification). This method has the disadvantage that the high molecular weight of the cell wall fractions makes them insoluble and the solution heterogeneous. Another method, which consists of mixing yeast cell wall and bacteria with a subsequent centrifugation and counting of non-bound bacteria in the supernatant, has some disadvantages, such as non-specific binding of bacterial cells during centrifugation (Eshdat et al., 1981). In addition, anti-adhesion agents are studied by means of their ability to interfere with the binding of bacteria to epithelial host cells (Eshdat et al., 1981; Oyofe et al., 1989). However, these methods cannot be suggested as

Table 1
Quantity of bound bacteria by 10 μg cell wall of *Trichosporon mycotoxinivorans*.

Strain	Source	Amount of bound bacteria: CFU/10 μg
<i>E. coli</i> 08 K88 F4 *Bio 104	AGES Vienna, Austria	4×10^3
<i>E. coli</i> 0139 K82 *Bio 108	AGES Vienna, Austria	1×10^3
<i>E. coli</i> 0149 K91 *Bio 109	AGES Vienna, Austria	*N.B.D.
<i>E. coli</i> 0108K—*Bio 107	AGES Vienna, Austria	1×10^2
<i>E. coli</i> 0141 K85ac *Bio 110	AGES Vienna, Austria	4×10^2
<i>E. coli</i> 0138 K81 *Bio 105	AGES Vienna, Austria	2×10^2
<i>E. coli</i> 0147 K89 *Bio 106	AGES Vienna, Austria	*N.B.D.
<i>E. coli</i> Nissle 1917*	Mutaflor, Ardeypharm	2×10^2
<i>E. coli</i> Nissle 1917 *Bio 694	Germany	
<i>Salmonella enterica</i> subsp. enterica *Bio 99	DSMZ 5569, Braunschweig, Germany, ATCC 13311	2×10^3
<i>Salmonella enterica</i> subsp. enterica *Bio 101	DSMZ 10506, Braunschweig, Germany	6×10^4
<i>Salmonella enterica</i> subsp. enterica *Bio 103	DSMZ 50912, Braunschweig, Germany	8×10^4
<i>Salmonella enterica</i> subsp. enterica *Bio 102	DSMZ 11320, Braunschweig, Germany	2×10^4
<i>Salmonella enterica</i> subsp. enterica *Bio 100	DSMZ 9274, Braunschweig, Germany	1×10^2
<i>Salmonella</i> Typhimurium S3	AGES Graz, Austria	2×10^4
<i>Salmonella</i> Typhimurium *Bio 60	USDA, Texas	1×10^4
<i>Salmonella</i> Enteritidis *Bio 59	USDA, Texas	6×10^3
<i>Salmonella</i> Choleraesuis *Bio 63	AGES Graz, Austria	2×10^2
<i>Campylobacter jejuni</i> SS LIO 103-920305 *Bio 66	CCUG 25903, Göteborg, Sweden	*N.B.D.
<i>Clostridium perfringens</i> 0307021 Type A *Bio 68	CCUG 47895, Göteborg, Sweden	*N.B.D.
<i>Lactobacillus salivarius</i> *Bio 505	BIOMIN isolate; DSMZ 16351	*N.B.D.
<i>Enterococcus faecium</i> *Bio 589	BIOMIN isolate; DSMZ 19746	*N.B.D.
<i>Salmonella</i> Choleraesuis *Bio 648	BIOMIN isolate; DSMZ 21288	*N.B.D.
<i>Bifidobacterium animalis</i> *Bio 503	BIOMIN isolate; DSMZ 16284	*N.B.D.
<i>Bifidobacterium thermophilum</i> *Bio 595	BIOMIN isolate; DSMZ 19756	*N.B.D.

*Bio Nr. = BIOMIN Research Center culture collection.

*N.B.D. = No binding detected.

routine based quality controls tools, as visual examinations are strongly operator dependent and cell culture techniques are very time-consuming, operator dependent and labor intensive. Most of the methods previously described are rather qualitative, as slight differences in the pathogen binding capacity between cell wall products are not detectable. The three-dimensional structure of mannose chains within the yeast cell wall is a critical feature to be taken into consideration; we propose that this has strong influence on the binding behaviour of different yeast cell wall products and is therefore presumed to be important for pathogen immobilization rather than the total amount of mannan. Indeed, quantitative, rather than qualitative assays are essential to evaluate the pathogen binding capacity of cell walls from different yeast sources and strains (Ganner et al., 2008).

Sokurenko et al. (1995) and Becker et al. (2007) used an experimental approach based on the optical density as a measure of adhering bacteria. The yeast cell wall represents a complex matrix of glycoconjugates which have high molecular weights. The microplate based assay used seemed to be a suitable and accurate tool since the insolubility of the cell wall material, as described earlier, can be taken as advantage and used

Fig. 3. Delayed growth curves of *E. coli* F4 bound to different concentrations of yeast cell wall: 10 µg yeast cell walls bind 4×10^5 CFU, 1 µg yeast 4×10^2 CFU, 0.1 µg yeast 4×10^1 CFU and 0.01 µg yeast 4 CFU; Binding control: consists of BSA and *E. coli* F4 and includes all assay steps; determines the bacterial number bound to non-specific binding sites; Growth control: no yeast product, only *E. coli* F4.

for coating the carrier. Sokurenko et al. (1995) developed an easily applicable method for measuring adherent bacteria based on the subsequent growth of the bacteria in a nutrient broth. However, in contrast to the current approach and to the approach of Becker et al. (2007), Sokurenko et al. (1995) recorded optical density readings not kinetically, but only did a single optical density reading, namely after 2–3 h of incubation time assuming to have reached the exponential growth phase of the bacteria. The three-dimensional structure of yeast cell wall and mannan is yet to be illuminated, because the coating-

Fig. 4. Binding of *S. Typhimurium* to a yeast cell wall product: 10 µg yeast cell walls bind 1×10^4 CFU, 1 µg yeast 1×10^3 CFU; Binding control: consists of BSA and *S. Typhimurium* and includes all assay steps; determines the bacterial number bound to non-specific binding sites; Growth control: no yeast product, only *S. Typhimurium*.

Fig. 5. Incubation of *L. salivarius* with yeast cell walls resulted in no binding; Binding control: consists of BSA and *L. salivarius* and includes all assay steps; determines the bacterial number bound to non-specific binding sites; Growth control: no yeast product, only *L. salivarius*.

reagents were limited to purified mannan molecules. It has been demonstrated by Ganner et al. (2007, unpublished data) and Ganner et al. (2008) that a pure standardized mannan could not bind pathogens such as *E. coli* F4; as a result we assume that the complex structure of yeast cell wall has strong influence on binding numbers of pathogens. Indeed these findings underline the importance of the application of yeast cell wall. Additionally, Sokurenko et al. (1995) monitored with an ELISA technique the attached bacteria using anti-*E. coli* antiserum resulting in an accurate and good correlation. Becker et al. (2007)

Fig. 6. Binding of *E. coli* Nissle 1917 to yeast cell wall: 10 µg yeast cell walls bind 2×10^2 CFU; 1 µg yeast binds 2×10^1 CFU; Binding control: consists of BSA and *E. coli* Nissle 1917 and includes all assay steps; determines the bacterial number bound to non-specific binding sites; Growth control: no yeast product, only *E. coli* Nissle 1917.

developed a similar microplate assay method for screening different binding matrices in food and feed ingredients. The assay is based on an inverse relationship between initial cell densities and the appearance of growth. The growth curves obtained were fitted by nonlinear regression analysis employing a sigmoidal curve model for a quantitative calculation. Afterwards, the evaluation was described by the kinetics of bacterial growth. This approach uses the correlation between optical density and the colony forming units counted on the agar plate for test development. In contrast to Becker et al. (2007), in the current study the equation of linear regression is used to determine the binding capacity of the yeast cell wall product. Baranyi and Pin (1999) previously described a numerical method to estimate bacterial growth parameters by means of detection times generated by different initial counts. However, Becker et al. (2007) did not focus on binding capacity and evaluation of yeast cell wall fractions for application in animal nutrition or on adhesion studies to various enteropathogenic bacteria and lactobacilli. There was a clear demonstration in the current research of the direct correlation between yeast cell wall concentrations and binding behaviour of pathogenic and non-pathogenic bacteria. The polystyrene surface of the microplate ensures the high binding capacity of the yeast cell wall fractions. The non-specific binding sites were subsequently blocked with bovine serum albumin (BSA) to demonstrate the specific binding between yeast cell wall and bacteria. The binding control consisting of BSA and test bacteria (without yeast cell wall, including all assay steps), in doing so, prevented growth in all assays until after 6 h. It is important to point out that the binding control, the bacterial number, which adhered to the non-specific binding sites of the well, was subtracted from the calculated data to determine the final number of specifically adhering bacteria. Bacterial growth as a measurement for adhesion to yeast cell wall has the advantage that it is easy, simple and does not require specific reagents such as stains, fluorescence or radioactive labels, or specific antibodies. The assay allows a high and convenient throughput.

The pathogenic bacteria investigated were chosen because they are known as infective agents of either animals or food-related infections in human. Zoonoses, which can be transferred from animal to human, can be a serious public health problem and furthermore, animals with enteric infections cause considerable economic losses in livestock farming. Indeed, the adherence of bacteria on mucosal tissues is an important step in the infectious process. In the past five decades, carbohydrate-binding proteins used by pathogens to recognize and adhere to cell surfaces have been described with increasing frequency (Zopf and Roth, 1996). One way pathogens bind to epithelial cells is through attachment of Type-I mannose-specific lectin-like fimbriae (Ofek et al., 1977). Several studies have reported *E. coli* and *Salmonella* strains as well as lactobacilli binding to intestinal epithelial cells via this mechanism (Korhonen et al., 1981; Ofek and Beachey, 1978; Ofek et al., 1977), however little is known about how probiotic coliform bacteria bind to the intestinal wall. *In vitro* agglutination results suggest that adsorption of *Salmonella* and *E. coli* by mannan-oligosaccharides could keep them from adhering to the intestinal wall. Yeast cell wall components, like mannan-oligosaccharides, have been shown by different authors to bind pathogenic bacteria such as *E. coli*, *Salmonella* strains and *Vibrio cholerae* with the *in vitro* agglutination method and visual examination (Eshdat et al., 1981; Mirelman et al., 1980; Oyofe et al., 1989; Peng et al., 2001; Spring et al., 2000). Spring et al. (2000) reported the cecal reduction of *S. Typhimurium* and *S. Dublin* by adding yeast cell wall (MOS) to the diet. Oyofe et al. (1989) also reported, in accordance with Spring et al. (2000), decreased cecal colonization of *S. Typhimurium* in young chicks when fed yeast cell wall in the diet. Duguid et al. (1966) reported a high frequency of Type-I fimbriae expression in *S. Typhimurium*, *S. Typhi* and *S. Enteritidis*. However, Mirelman et al. (1980) only found 4 of 13 strains of *S. Typhimurium* to express Type-I fimbriae. Our findings agree with those of Duguid et al. (1966), Spring et al. (2000) and Ofek et al. (1977) that yeast cell wall has high binding properties for *S. Typhimurium*,

S. Enteritidis and enteropathogenic *E. coli* strains. However, the strain and serovar selectivity also needs to be considered. Indeed we are limited with our method to explain whether Type-I fimbriae cause the binding to the yeast cell wall or any other unknown factors may be involved.

Spring et al. (2000) investigated the agglutination of five *C. jejuni* strains (visual examination); four of them did not show any agglutination to the yeast cell wall, one of them a weak agglutination; however, as has already been noted, visual examination and qualitative evaluation is not sufficient. McSweeney and Walker (1986) investigated mechanisms on the adherence of *C. jejuni* to epithelial cells *in vitro*. The adherence of *C. jejuni* to cells was partially inhibited by adding certain carbohydrates (fucose or mannose) to the bacterial cells. Schoeni and Wong (1994) described protective effects of dietary mannose against *C. jejuni* colonization in chicken. More importantly, our method clearly describes no growth of *C. jejuni* as a consequence of its not being able to bind to the cell wall. Again, this shows that some other binding mechanisms not yet fully understood described by the different authors may be involved.

Mannose-specific adherence mechanisms of lactobacilli have been described (Adlerberth et al., 1996). Adlerberth et al. (1996) demonstrated that certain strains of *Lactobacillus plantarum* have a mannose-specific adherence mechanism. However, no binding to the mannose-containing yeast cell wall by the current selected strains of lactobacilli and bifidobacteria could be clearly demonstrated in contrast to the *Salmonella* and *E. coli* strains. Conversely, the probiotic coliform *E. coli* Nissle strain 1917 is bound by the yeast cell wall with similar amounts as for the enteropathogenic *E. coli* strains.

Our results reveal and confirm that yeast cell wall has a selective effect against some pathogenic bacteria, while having no adverse effect on beneficial and commensal bacteria.

5. Conclusion

A novel routine tool for the feed industry has been developed. The microplate based *in vitro* test system is an accurate and user-friendly tool to investigate the quantitative binding capacity of yeast cell wall against enteropathogenic bacteria as well as non-pathogenic lactobacilli and bifidobacteria. Selective binding to specific bacterial strains could be clearly demonstrated. Further studies of a wider range of *E. coli* and *Salmonella* strains and of other enterobacteria from different pathovars as well as studies on cell culture models would also be helpful. The currently suggested binding mechanisms involved are not fully understood; either through mannose specific Type-I fimbriae or other unknown mechanisms. For this purpose, studies on a molecular level and detailed characterization of cell wall components have to be examined in future research studies.

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Erratum

Erratum to “Quantitative *in vitro* assay to evaluate the capability of yeast cell wall fractions from *Trichosporon mycotoxinivorans* to selectively bind gram negative pathogens”

[J. Microbiol. Methods 83 (2010) 168–174]

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The publisher regrets that in the original printing of the above-mentioned paper, Table 1 was produced incorrectly. This error has now been corrected.

The publisher would like to apologise for any inconvenience this may have caused to the authors of this article and the readers of the journal.

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Strain	Source	Amount of bound bacteria: CFU/10 µg
<i>Escherichia</i>		
<i>E. coli</i> 08 K88 F4 *Bio 104	AGES Vienna, Austria	4×10^3
<i>E. coli</i> O139 K82 *Bio 108	AGES Vienna, Austria	1×10^3
<i>E. coli</i> O149 K91 *Bio 109	AGES Vienna, Austria	–
<i>E. coli</i> O108K *Bio 107	AGES Vienna, Austria	1×10^2
<i>E. coli</i> O141 K85ac *Bio 110	AGES Vienna, Austria	4×10^2
<i>E. coli</i> O138 K81 *Bio 105	AGES Vienna, Austria	2×10^2
<i>E. coli</i> O147 K89 *Bio 106	AGES Vienna, Austria	–
<i>E. coli</i> Nissle 1917*	Mutaflor, Ardeypharm	2×10^2
<i>Salmonella</i>		
<i>Salmonella enterica</i> subsp. <i>enterica</i> *Bio 99	DSMZ 5569, Braunschweig, Germany, ATCC 13311	2×10^3
<i>Salmonella enterica</i> subsp. <i>enterica</i> *Bio 101	DSMZ 10506, Braunschweig, Germany	6×10^4
<i>Salmonella enterica</i> subsp. <i>enterica</i> *Bio 103	DSMZ 50912, Braunschweig, Germany	8×10^4
<i>Salmonella enterica</i> subsp. <i>enterica</i> *Bio 102	DSMZ 11320, Braunschweig, Germany	2×10^4
<i>Salmonella enterica</i> subsp. <i>enterica</i> *Bio 100	DSMZ 9274, Braunschweig, Germany	1×10^2
<i>Salmonella</i> Typhimurium S3	AGES Graz, Austria	2×10^4
<i>Salmonella</i> Typhimurium *Bio 60	USDA, Texas	1×10^4
<i>Salmonella</i> Enteritidis *Bio 59	USDA, Texas	6×10^3
<i>Salmonella</i> Choleraesuis *Bio 63	AGES Graz, Austria	2×10^2
<i>Campylobacter</i>		
<i>Campylobacter jejuni</i> SS LJO 103-920305 *Bio 66	CCUG 25903, Göteborg, Sweden	*N.B.D.
<i>Clostridium</i>		
<i>Clostridium perfringens</i> 0307021 Type A *Bio 68	CCUG 47895, Göteborg, Sweden	*N.B.D.
<i>Lactobacilli</i>		
<i>Lactobacillus salivarius</i> *Bio 505	BIOMIN isolate; DSMZ 16351	*N.B.D.
<i>Enterococcus faecium</i> *Bio 589	BIOMIN isolate; DSMZ 19746	*N.B.D.
<i>Lactobacillus reuteri</i> *Bio 648	BIOMIN isolate; DSMZ 21288	*N.B.D.
<i>Bifidobacteria</i>		
<i>Bifidobacterium animalis</i> *Bio 503	BIOMIN isolate; DSMZ 16284	*N.B.D.
<i>Bifidobacterium thermophilum</i> *Bio 595	BIOMIN isolate; DSMZ 19756	*N.B.D.

*Bio Nr. = BIOMIN Research Center culture collection.

*N.B.D. = No binding detected.

**Ex vivo effect of yeast beta-glucan on lymphocyte viability
and plasma IL-18 in weaning piglets**

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Ex vivo effect of yeast beta-glucan on lymphocyte viability and plasma IL-18 in weaning piglets[☆]

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ABSTRACT

Beta-glucans have been considered as potential growth promoters due to modifying the immune system. The trial objective was to study the efficacy of beta-glucan on lymphocyte viability and plasma IL-18 of weaning piglets. Four experimental groups with the following trial design were included in the study: control group A without beta-glucan, group B, C and D with 100, 250 and 500 mg/kg of beta-glucan, respectively. Blood samples of 6 piglets per group were taken on day 7 and 21. Lymphocytes were separated with density gradient centrifugation. WST-1 assay was used to determine lymphocyte viability and ELISA to quantify plasma IL-18. The beta-glucan preparation had an elevating effect on lymphocyte viability in doses of 100 mg/kg on day 7 and in doses of 250 mg/kg and 500 mg/kg on day 21. IL-18 showed a slight increase on day 21 in all trial groups in comparison to the control. It can be concluded, that yeast beta-glucan might be used as an immunomodulating agent.

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1. Introduction

The ban of antibiotic growth promoters in the European Union in 2006 stimulated research in the field of functional feed additives to find natural alternatives. Yeast derived beta-glucans have been considered as potential growth and health promoters by beneficially modifying the immune system. Cells of the innate immunity such as macrophages, phagocytes and natural killer cells have surface beta-glucan receptors, which specifically recognize and bind the beta-1,3-glucan linkage of the beta-glucan molecule. Lymphocytes belong to the acquired immunity and play a key role in defending the body against disease.

Numerous studies, summarized by Kogan and Kocher (2007), have shown that yeast beta-glucan modulates the innate immunity and enhances the functional status of

macrophages, stimulates the release of cytokines and exerts anti-tumor as well as antioxidant activity. Li et al. (2005) reported that dietary beta-glucan attenuated the increase of pro-inflammatory cytokines (IL-6, TNF-alpha) whereas the increase of IL-10 was potentiated in an *in vivo* study with LPS-challenged weaning piglets. However, in another study Li et al. (2006) observed a trend towards elevated pro-inflammatory (IL-6 and TNF-alpha) plasma cytokines in LPS-challenged piglets. Mao et al. (2005) observed improved lymphocyte proliferation of LPS-challenged pigs fed with beta-glucan.

Pro-inflammatory cytokines are among the first cytokines produced in response to pathogenic bacteria (Miettinen et al., 1998). IL-18, also known as IFN-gamma activating factor, is an important regulator of innate and acquired immune responses and has also been described to enhance host defense (Dinarello, 1999, 2000). IL-18 produced by activated macrophage-like cells acts synergistically with IL-12 by enhancing IFN-gamma gene expression in T-cells (Miettinen et al., 1998). Previously it has been demonstrated that both, IL-12 and IL-18 promote inflammatory response; however there is a post-weaning decrease in gene expression of piglets reflecting either an adaptive immune response to the

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weaning transition or a consequence of the maturation of the intestinal immune system (Pie et al., 2004).

Our trial objective was to study the efficacy of yeast beta-glucan on *ex vivo* lymphocyte viability and plasma IL-18 of weaning piglets.

2. Material and methods

Twenty-four, 4 week-old mixed-sex weaning piglets were divided into 4 experimental groups with 3 replicates each and were fed 0, 100, 250, and 500 mg beta-glucan/kg feed, respectively. Blood samples of 6 piglets per group were taken on days 7 and 21 after adding beta-glucan to the diet. Lithium-heparin tubes were used. Heparin tubes were centrifuged and plasma was stored at -80°C . Lymphocytes were separated from the whole blood with histopaque, which is a solution of polysaccharides with a density of 1.077 in order to facilitate isolation of viable lymphocytes. A density of 10^6 cells/mL was pipetted into a 96 well plate and the WST-1 (water soluble tetrazolium salt) assay was used to determine cell viability (Roche Diagnostics, Austria). The principle of this assay is that tetrazolium salts are transformed to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates with the number of metabolically active cells. Quantification of the formazan dye produced by metabolically active cells was done by a scanning multi-well spectrophotometer. The absorbance of the dye solution was measured at a wavelength of OD₄₅₀. ELISA-assay (Biosource) was used to quantify plasma IL-18. Statistical analyses were performed with SPSS 15.0; analysis of variance (ANOVA) and Tukey test were used to evaluate the data.

3. Results and discussion

Controversial data have been published regarding the immunomodulating effect of yeast derived beta-glucans. A contribution to this research field has been tried to approach focusing on the viability of lymphocytes and the IL-18 modulation.

On day 7, lymphocyte viability from pigs fed 100, 250 or 500 mg/kg beta-glucan was not statistically significantly different from those of control pigs ($P=0.28$). However, slightly enhanced lymphocyte viability from pigs fed 100 mg/kg (OD 0.73) and 500 mg/kg (OD 0.68) dietary beta-glucan was noticed in comparison to the control (OD 0.62; Fig. 1a). On day 21, pigs fed 250 or 500 mg/kg beta-glucan had higher (both OD 0.61; $P=0.003$) lymphocyte viability than the control (OD 0.48; Fig. 1b).

Although not significantly ($P=0.29$), the pro-inflammatory cytokine IL-18 was slightly enhanced in all trial groups (100 mg/kg: 682 pg/mL; 250 mg/kg: 667 pg/mL; 500 mg/kg: 751 pg/mL) on day 21 in comparison to the control (616 pg/mL plasma) (Fig. 2). The only slight difference in lymphocyte viability between the control group and the trial groups as well as the slightly enhanced pro-inflammatory IL-18 levels might be due to lack of bacterial challenge in this trial, which has been carried out under clean experimental conditions.

Fig. 1. a: Lymphocyte viability on day 7 after addition of beta-glucan to the diet ($P=0.28$). b: Lymphocyte viability on day 21 after addition of beta-glucan to the diet. * $P<0.05$ compared to control.

Furthermore, individual or gender-specific differences are quite substantial in weaning piglets; this could be minimized with higher random sampling numbers. Regarding beneficial effects on lymphocytes and elevated pro-inflammatory cytokines our results are in accordance with Li et al. (2006)

Fig. 2. Plasma IL-18 on day 21 after addition of beta-glucan to the diet ($P=0.29$).

and Mao et al. (2005). Indeed, we made different observations than Li et al. (2005), which observed that dietary beta-glucan preparations attenuated the increase of pro-inflammatory cytokines. Towards different observations in literature regarding elevated or attenuated pro-inflammatory cytokines, we assume that the structure, the source and the branches of beta-glucan have substantial impact on immunological functions in dietary treatments (Li et al., 2005, 2006). As there are not many studies devoted to enhance the lymphocyte viability by beta-glucan preparations, we consider the present study as an initial step in this research area. However, the observed trend towards increased lymphocyte viability needs further elucidation. Furthermore, a challenge study would be illuminating regarding the pro-inflammatory and immunomodulating cytokine IL-18.

4. Conclusion

In summary, the beta-glucan preparation had a beneficial effect on lymphocyte viability at dosage levels 250 mg/kg and 500 mg/kg on day 21. IL-18 showed a slight increase at all dosage levels on day 21 in comparison to the control. Yeast beta-glucan might be considered as an immunomodulating agent which may fortify animal health in critical transition periods.

Conflict of interest

There is no conflict of interest concerning people or organizations. Anja Ganner, Alfred Klimitsch, Gerd Schatz-

mayr, Kathrin Erlacher belong to BIOMIN Research Center. Sabine Nitsch belongs to BIOMIN Holding.

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**Evaluation of yeast derivatives to bind *E. coli* F4
quantitatively**

Evaluation of yeast derivatives to bind *E. coli* F4 quantitatively

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Abstract

Target of the present study was the evaluation of commercially available yeast derivatives for its capacity to bind *E. coli* F4 quantitatively. Mannan and glucan content of the products were compared statistically with the binding numbers. In a subsequent experiment with fermentation and autolysis of different yeast strains, influence of yeast strains were put into correlation with the *E. coli* F4 binding numbers. Capacity of yeast cell wall products to bind *E. coli* F4 was assessed with a quantitative microbiological microplate-based assay by measuring the optical density as growth parameter of adhering bacteria. Total mannan and glucan were determined by HPLC using an isocratic method and a Refractive Index (RI) Detector. Statistical analyses were performed with SPSS (v10). Different cell wall and autolysate products show different binding numbers, that means differences in the product quality. No direct correlation between total mannan nor mannoprotein content and binding numbers; we consider the mannan structure as an important factor for pathogen immobilization. Variable binding numbers were also noted by different yeast strains, *Trichosporon mycotoxinivorans* (MTV) shows highest binding number. We believe that the cell wall composition and structure plays an important role in pathogen immobilization, rather than just the total amount of mannan; besides that we assume that unknown factors influence the binding capacity of yeast derivatives.

Keywords: yeast cell wall, *E. coli* F4, microplate-based assay

Introduction

Yeast derivatives such as yeast cell wall products and recently also yeast autolysate products are sold worldwide as feed supplements with health claims to bind enteropathogenic bacteria such as *E. coli* and *Salmonella* spp.. Although yeast cell wall products have been fed for a long time, their binding capacity and their mechanism on how to bind pathogens is still not well understood. “Not all yeast products are the same” and there is huge confusion throughout the industry concerning the quality of yeast products. An important initial event in bacterial pathogenesis is the adherence of bacteria via their surface lectins to host intestinal cells. Infections are initiated only after the microorganism has first adhered to the host cell surface. If adherence can be inhibited then the subsequent infection can also be inhibited. This approach forms the basis of anti-adherence strategies. The most studied are receptor-analogs, which include oligosaccharides (Shoaf-Sweeney and Hutkins, 2008).

According to literature mannan-oligosaccharides (MOS) are the substances in the yeast cell wall which are responsible for binding of pathogens (e.g. Snellings *et al.*, 1997; Sharon *et al.*, 2000). However, recently it has been questioned if the amount of mannoprotein in the yeast cell wall is the compound responsible for immobilizing enteric pathogens. A weak point so far was the lack of quantitative analytical methods to determine the total amount of bacteria bound by yeast cell wall (Applegate *et al.*, 2010). Some qualitative test procedures such as the agglutination method are available to investigate the binding capacity of yeast products (e.g. Mirelmann *et al.*, 1980; Eshdat *et al.*, 1981; Oyofe *et al.*, 1989; Spring *et al.*, 2000; Peng *et al.*, 2001). However, quantitative measurement of the bacterial adhesion is necessary to evaluate the binding capacity and the quality of yeast derivatives.

The quantitative *in vitro* assay developed by Ganner *et al.* (2010) has been used in the present study to investigate different yeast cell wall products (MOS) and yeast autolysates to bind *E.*

coli F4 quantitatively. *E. coli* F4 has been chosen for the present study as it is an important pathogen in animal sciences and is known to cause severe economic losses in swine production. Furthermore, mannan, glucan and protein content were determined of the yeast products and statistical evaluation for correlation performed with SPSS v10.

To get more information regarding binding characteristics, apart from mannan and glucan content, in a separate experiment, lab scale fermentations and autolysis have been performed with 6 different yeast strains used in the feed industry to investigate the potential influence of yeast strains.

Material and Methods

Yeast derivatives

Different commercially available yeast cell wall and autolysed yeast products were used. Due to confidential agreement trade name and companies are not mentioned (sample key is hided).

Autolysis of cell wall fragments

Cell wall fragments were produced in laboratory scale (standardized conditions) by the following yeast strains: *Saccharomyces cerevisiae*, Bio 19* (DSMZ 1848), *Klyveromyces marxianus* Bio 21* (DSMZ 5420), *Saccharomyces boulardii* CAN 214*, *Saccharomyces boulardii* HA282*, *Saccharomyces boulardii* HA283*, *Trichosporon mycotoxinivorans* (MTV)*.

Cell wall fragments were obtained by incubating the respective yeast strain in 1000 ml shaking flask containing 400 ml medium consisting of 1% soy peptone, 0.1% yeast extract, 0.2% magnesium sulfate heptahydrate, 0.2% ammonium sulfate, 1% saccharose and 2.5%

glucose for 48 hours at 30°C and pH 5.0. Subsequently yeast cells were autolysed by incubation at pH 5, at 50°C for 24 hours and centrifuged (17700×g, 10 min) to separate cell wall fragments from the supernatant. Finally the cell wall fragments were freeze dried (24 hours, -30°C/+30°C, 0.001 mbar) (Epsilon 2-90, Christ, Osterode, Germany).

Autolysis was adapted according to Liu *et al.* (2008); the degree of cell rupture was monitored by increasing amount of protein- and carbohydrate content as well as dry matter in the supernatant. Peak protein and carbohydrate concentration as well as dry matter mass were determined after 24 hours. Additionally the progress of autolysis was observed under the microscope (1000 times magnification) and compared to the original culture.

*BIOMIN Research Center culture collection

Microplate-binding assay

The *in vitro* capacity to bind *E. coli* F4 was determined with a quantitative microbiological microplate-based assay by measuring the optical density as growth parameter of adhering bacteria. The yeast derivatives were suspended in phosphate buffered saline (0.01%), aliquots of 100 µL/well were pipetted in a 96-well plate and incubated for 16-18 hours at 4°C. With the microplate washer (Tecan 5082 M8/4R Columbus Plus)/PBSTween20 the plate was washed three times. Subsequently, the bacterial suspension of *E. coli* F4 was adjusted to OD 0.01 and added in the wells of the microplate. Bacteria were allowed to adhere for 60 minutes at 37°C and afterwards washed 6 times with microplate washer/PBSTween to remove non-adherent bacteria. The wells were filled with 200 µL of TSB and coated with one drop paraffin-oil. The microplate was placed in a microplate reader (Tecan Genius), incubated for 18 hours at 37°C and an OD of 690 nm. Data were recorded every 15 minutes (according to Ganner *et al.*, 2010).

Carbohydrate analysis

Total mannan and glucan were determined by HPLC using an isocratic method and a Refractive Index (RI) Detector. Polysaccharides were hydrolyzed with 72% sulfuric acid; with a subsequent Carrez-precipitation interfering materials were removed. Using the Carrez-precipitation distracting proteins and fats are precipitated as zinc (2+) and/or cyanoferrat (II) - complexes (Matissek *et al.*, 1992).

Statistical analysis

The data were analyzed using SPSS, version 10. Bi-variat correlations were analyzed by Pearson correlation coefficient. Coefficient of correlation (r) was either expressed as positive (+) or negative (-). The closer the coefficient is to either -1 or 1, the stronger is the correlation between the variables.

Results and Discussion

Different commercially available yeast cell wall products, autolysate products and beta-glucan products have been tested for their ability to bind *E. coli* F4 quantitatively. The different cell wall products bound variably amount of *E.coli* F4, however binding capacity was not dependent on the total amount of neither mannan nor mannoprotein (Table 3, Table 4). Cell wall product 4 with 26% mannose content bound 1×10^4 CFU/mg while cell wall product 5 with only 9% mannan bound 8×10^5 CFU/ml. In contrast, beta-glucan did not bind any bacteria.

There is plenty of literature which describes adherence mechanisms and proposes mode of action of yeast derivatives (Mourao *et al.*, 2006; Oyofe *et al.*, 1989; Sharon and Ofek, 2000;

Shoaf-Sweeney and Hutkins, 2008; Snellings *et al.*, 1997; Spring *et al.*, 2000; Terre *et al.*, 2007; Yang *et al.*, 2008). However, no study has been devoted before to investigate to quantitative amount of pathogens bound by different yeast derivatives. Yeast strain influence has also not been investigated before in combination with pathogen binding. We consider this as an important quality parameter for yeast cell wall products. Indeed there was a lack of quantitative analytical methods to determine the total amount of bacteria bound by yeast cell wall (Applegate *et al.*, 2010). Yeast cell wall products are suggested as anti-anti-adhesive agents and thus proposed to prevent intestinal bacterial attachment by providing alternative adhesion sites to enterobacteria which contain mannose-specific type I fimbriae such as *E. coli* and *Salmonella* spp. (Ofek *et al.*, 1977). Shoaf-Sweeney and Hutkins (2008) describe that the adhesion affinity is defined by the valency of protein-oligosaccharide interactions. A single molecule would have a low affinity, but the creation of individual protein subunits with numerous oligosaccharide binding sites assembled to a filamentous structure leads to increased target avidity. In previous experiments we could underline the speculation of Shoaf-Sweeney and Hutkins (2008), a purified mannan standard (99%) showed in contrast to yeast cell wall products (mannan content between 10-25%) no pathogen binding activity (Ganner *et al.*, 2007, unpublished data). However, no correlation neither with mannan-protein ($r = -0.485$) nor by the ratio of mannan to protein ($r = 0.077$) in the present experiment.

The three-dimensional structure of mannose chains within the yeast cell wall is a critical feature to be taken into consideration; we propose that this has strong influence on the binding behaviour of different yeast cell wall products and is therefore presumed to be important for pathogen immobilization rather than the total amount of mannan. Indeed, quantitative, rather than qualitative assays are essential to evaluate the pathogen binding capacity of cell walls from different yeast sources and strains (Ganner *et al.*, 2008). Ofek *et al.*, (2003) describes

that apart from adhesion-receptor interaction, hydrophobic and other non-specific interactions under different share-forces might be involved in the adhesion process.

Becker and Galetti (2008) investigated various food and feed components for alternative adhesion of intestinal *E. coli* and *Salmonella*. Yeast mannan-oligosaccharides, pumpkin, sesame seed extract, palm kernel extract and konjac gum were among the strongest binding matrices for enteropathogens. Adhesion matrix differ tremendous, this makes interpretation and speculation about binding factors substantial. Thus, indication is given that unspecific, unknown binding properties are involved. Moreover, taking the diversity of natural products, due to biological variation for processing, into account, the binding capacity of fibrous food and feed components for different bacteria seems rather unpredictable (Becker and Galetti, 2008)

We could find, according to literature, that mannan content is strain dependent (Table 1); *Trichosporon mycotoxinivorans* contains less mannan than *Saccharomyces* spp. (Jaehrig *et al.*, 2008; Klis *et al.*, 2002; Molnar *et al.*, 2004), however highest binding number (Table 2). Statistically there is no correlation between yeast strain and binding capacity to *E. coli* F4 (Table 4). Due to the fact that strain *Trichosporon mycotoxinivorans* with lowest mannan content and highest binding number, we have a high negative correlation coefficient ($r = -0.71$). It can be speculated about the involved binding mechanism between pathogens and yeast cell wall; maybe production process, yeast cell wall composition, cell wall structure, strain diversity etc. are some of the influencing factors. Again, we believe that the cell wall structure and the structural surroundings apart from non-specific interactions such as hydrophobic and other share forces play important roles in pathogen immobilization, rather than just the total amount of mannan.

Giovanni *et al.* (2009) studied the effect of yeast strain and fermentation conditions on the release of cell wall polysaccharides. Temperature, media and sugar composition influenced

the release of cell wall polysaccharides significantly. Thus, again underlines and supports the speculations about the relative influence of production process and production conditions on binding capacity

Conclusion

The present study demonstrates that there is a huge variation between different yeast products concerning their capability to bind *E. coli* F4 quantitatively. Indeed there is no direct correlation neither between the total mannan content nor the mannoprotein content and the binding numbers. Thus, the currently suggested binding mechanisms involved are not fully understood; either through mannose specific type-I fimbriae or further, synergistic, unknown mechanisms. Biological variation and natural diversity make prediction even more difficult and comprehensive. In any case further research is necessary, e.g. on a molecular level and a detailed characterization of cell wall components and cell wall structure, to elucidate which additional forces might be involved in the binding mechanism between yeast derivatives and *E. coli* F4. Furthermore, more standardized *in vivo* experiments with challenged animals would also be illuminating to validate the *in vitro* binding potential of yeast products and to study the anti-adherence strategy.

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Figure captions

Table 1: Capability of yeast derivatives to bind *E. coli* F4; mannan, glucan and protein content

Table 2: Influence of different yeast strains (standardized laboratory fermentation and autolysis conditions) on *E. coli* F4 binding capability; mannan-and glucan content

Table 3: Correlation between quantity of bound *E.coli* F4 (CFU/mg) and mannan, glucan, protein content of yeast derivatives

Table 4: Correlation between quantity of bound *E.coli* F4 (CFU/mg) and mannan, glucan content of different yeast strains (fermented under standardized laboratory condition)

Table 1: Capability of yeast derivatives to bind *E. coli* F4; mannan, glucan and protein content

Yeast derivative	Quantity of bound <i>E. coli</i> F4 (CFU/mg)	Mannan content*	Glucan content*	Protein content*
cell wall 1	40.000, 24.000, 35.000	20%	37%	25%
cell wall 2	40.000, 60.000, 100.000	13%	29%	25%
cell wall 3	400.000, 230.000, 340.000	26%	27%	25%
cell wall 4	12.000, 6.000, 12.000	26%	27%	31%
cell wall 5	480.000, 2000.000, 700.000	9%	25%	27%
cell wall 6	60.000, 14.000, 13.000	7%	28%	30%
cell wall 7	160.000, 140.000, 140.000	25%	25%	30%
autolysate 1	260.000, 220.000, 140.000	5.5%	12%	35%
autolysate 2	20.000, 34.000, 33.000	15.5%	22%	40%
autolysate 3	20.000, 8000, 10.000	16%	25%	41%
autolysate 4	20.000, 8000, 40.000	14%	28%	45%
autolysate 5	20.000, 10.000, 60.000	19%	28%	40%
autolysate 6	2000, 8000, 20.000	13%	24%	36%
Yeast derivative 1	-	1%	67%	4%
Yeast derivative 2	-	1%	60%	5%

*Dry matter

Table 2: Influence of different yeast strains (standardized laboratory fermentation and autolysis conditions) on *E. coli* F4 binding capability; mannan-and glucan content

Yeast strain	Quantity of bound <i>E. coli</i> F4 (CFU/mg)	Mannan content	Glucan content
<i>Saccharomyces cerevisiae</i> Bio19 (DSMZ 1848)	12.000, 30.000	20%	16%
<i>Kluyveromyces marxianus</i> Bio21 (5420)	4400, 36000	17%	20%
<i>Saccharomyces boulardii</i> CAN 214*	1000, 1800	24%	14%
<i>Saccharomyces boulardii</i> HA282*	96.000, 32.000	20%	12%
<i>Saccharomyces boulardii</i> HA283*	60.000, 16.000	24%	17%
<i>Trichosporon mycotoxinivorans</i>	78.000, 120.000	12%	30%

*BIOMIN Research Center culture collection

Table 3: Correlation between quantity of bound *E.coli* F4 (CFU/mg) and mannan, glucan, protein content of yeast derivates

	Mannan content	Glucan content	Mannan / Glucan	+ Mannan / Protein	+ Protein / Mannan	/ Glucan / Mannan
correlation (Pearson)	-0.23	-0.18	-0.276	-0.485	0.077	0.229
samples (N)	12	12	12	12	12	12

Table 4: Correlation between quantity of bound *E.coli* F4 (CFU/mg) and mannan, glucan content of different yeast strains (fermented under standardized laboratory condition)

	Mannan content	Glucan content	Mannan + Glucan	Mannan / Glucan
correlation (Pearson)	-0.71	0.632	0.226	-0.558
samples (N)	6	6	6	6

**Efficacy of a yeast derivative on broiler performance,
intestinal morphology and immunological blood
parameters**

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Efficacy of a yeast derivative on broiler performance, intestinal morphology and immunological blood parameters

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Abstract

The trial objective was to investigate the efficacy of a yeast derivative (YD) on performance, health status, distal jejunal structure, and blood profile of broiler chickens.

In a 35 d feeding trial, 1 d-old broilers were allocated to 3 experimental groups with 8 replicates (26/pen): control, treatment 1 (1 kg YD/t feed) and treatment 2 (2 kg YD/t feed). Feed and water were provided *ad libitum*. On d 35 birds were bled and euthanized and the distal jejunum collected from 8 chickens per group (1/pen) for morphometric measurements. Blood samples were collected (1/pen) and on d 35 and analyzed by flow cytometry for leukocyte, heterophil, lymphocyte and monocyte enumeration.

In the course of the trial a positive influence was observed by supplementation of YD. On d 35 body weight (BW) (1520 g) and daily weight gain (42.37 g/d) was greater in birds receiving 1 kg/t of YD compared to the control (1374 g BW, 38.18 g /d, respectively; $P=0.05$). Supplementation with 2 kg/t of YD did not improve 35 d BW but did improve cumulative feed-to-gain by 13% ($P<0.05$) versus birds fed the control diet. Histological evaluation demonstrated greater goblet cell density in birds fed either concentration of YD ($P<0.05$). Villus height and crypt depth, however, were unaffected. Apoptotic enterocytes were decreased by both concentrations of YD ($P=0.02$). Supplementation of YD had no affect

on blood cell counts ($P>0.05$). The inclusion of YD in diets of broilers was able to improve broiler performance, intestinal goblet cell numbers and to reduce numbers of enterocytes undergoing apoptosis.

Keywords: Blood cells; Broiler performance; Intestinal morphology; Yeast derivative

Introduction

Yeast products have been fed to animals for more than hundred years, either in the form of yeast fermented mash produced on the farm, yeast by-products from breweries and distilleries, or yeast products commercially produced for animal feeding. However, their *in vitro* and *in vivo* mode of action, in improving animal performance is still not well understood. Live yeasts have been described as being capable of protecting the intestinal mucosa against invading microorganisms by being antagonistic to undesirable microorganisms (barrier effect), and by contributing to the stimulation and maturation of the host animals immune response (Fuller et al., 1989). Yeast cell wall components (e.g. mannanoligosaccharides) reportedly prevent colonization of pathogens in the intestinal tract by binding of pathogenic bacteria which have mannose-specific type-I fimbriae and by having prebiotic activity, which leads to an increase in number of beneficial bacteria such as lactobacilli and bifidobacteria (Ofek and Beachey, 1978; Firon et al., 1984; Gibson and Wang, 1994; Newmann, 1994; Zopf and Roth, 1996; Shoaf-Sweeney and Hutkins, 2009). β -glucans, a component of the yeast cell wall, have been shown to stimulate the immune response, for example by increasing numbers of phagocytic cells such as macrophages. Autolysed yeast has been proposed to possess dual beneficial effects such as immunomodulation and pathogen control/defense (Kogan and Kocher, 2007).

Mannanoligosaccharides, another yeast cell wall component, are used as a natural feed additive for poultry due to their purported intestinal health benefits. Baurhoo et al. (2007), Baurhoo et al. (2009) and Solis de los Santos et al. (2007) investigated mannanoligosaccharide preparations in chickens and observed improved gut morphology including longer villi, shorter crypts and enhanced goblet cell numbers in the villus. Beyond the surface area improvement, goblet cell numbers are critical due to production of intestinal mucus. Mucus has several functions such as lubricating intestinal surfaces, trapping and neutralizing bacteria, detoxifying heavy metals by binding, interacting with the intestinal immune system, acting as a diffuse barrier for nutrients and macromolecules thus protecting the underlying epithelial cells (Forstner and Forstner, 1994). Another important function of mucin is that the structure of the mucus provides several potential attachment sites and colonization niches for commensal and pathogenic bacteria (Sonnenburg et al., 2004).

Furthermore, yeast-derived polysaccharides, such as β -glucans, have been described to have the ability to modulate the immune system. The most active immune stimulators appear to be branch chained 1,3- β -D-glucans, sometimes referred to as 1,3- and 1,6- β -D-glucans, present in the yeast cell wall. These branch chain glucans have the capacity to activate the innate immune system, thereby enhancing the defense barriers and providing protection against a variety of infections (Raa, 1996; Stuyven et al., 2008). The mannanoligosacchride fraction can modulate, in part, the immune system by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Ferket et al., 2002). Heterophils, lymphocytes and monocytes are leukocytes which help the body to fight against infectious diseases and foreign substances. Heterophils and monocytes, which belong to the innate immune system, have the ability to phagocyte bacteria and parasites to protect the animal against primary infections. The heterophil is a highly phagocytic, granulated cell capable of antimicrobial activity and is analogous, although not identical, to the mammalian neutrophil (Harmon, 1998; Huff et al.,

2007). Huff et al. (2007) reported an increase in heterophil (percentage of leucocytes) in whole blood in a study in which turkeys were fed yeast extract and challenged with *E. coli*. While activation of heterophils is an important component of the innate immune response, heterophils are also responsible tissue damage by accumulation in inflamed tissue and forming heterophil granulomas that are morphological similar to inflammatory lesions in reptiles (Harmon, 1998). In mammals, stress and infection have been shown to increase the rate of neutrophil production by 10-fold (Smith, 1994). Numerous studies, summarized by Kogan and Kocher (2007), have shown that yeast derivatives, such as β -glucan fractions, enhance the functional status of macrophages; however, little is known about the effect of yeast derivatives on the number of monocytes, the precursor of macrophages.

The objective of the present study was to investigate a YD for its ability to influence broiler performance, intestinal characteristics (morphology, goblet cell numbers, and numbers of apoptotic enterocytes), and blood cell profile (monocytes, heterophils and lymphocytes). The YD used in this study was characterized by determining mannan-, glucan- and protein content. Furthermore the YD was characterized *in vitro* for its capacity to bind *Salmonella typhimurium* and *E. coli* F4 before use in the feeding trial.

Material and Methods

Experimental design. Day-old Ross 308 broiler chicks (mixed sex) were randomly assigned to one of three dietary treatments for the 35 d feeding period. Dietary treatments included feeding YD at 0, 1, or 2 kg/t diet to 8 replicate pens of 26 chicks per pen. Each pen was 1.32 m² and had clean wood shavings litter, nipple drinkers and automatic feeders. Climate conditions and the lighting program were controlled by a computer system according

to the standard recommendations for broiler chicks, and the changes in environmental conditions were automatically recorded daily. The diet formulation and calculated nutrient composition are shown in Table 1.

Yeast derivative. The yeast preparation used in the study contains yeast cell wall fragments and yeast extract derived from *Saccharomyces cerevisiae*. The YD was analyzed for mannan-, glucan- and protein content and the capacity to bind *Salmonella typhimurium* and *E. coli* F4 *in vitro*.

Total mannan and glucan were determined by HPLC using an isocratic method and a Refractive Index (RI) Detector. Polysaccharides were hydrolyzed with 72% sulfuric acid; with a subsequent Carrez-precipitation interfering materials were removed. Using the Carrez-precipitation distracting proteins and fats are precipitated as zinc (2+) and/or cyanoferrat (II) - complexes (Matissek et al., 1992).

Total protein was determined by Total Kjeldahl Nitrogen (TKN, AOAC Official Method 954.01).

The *in vitro* capacity to bind *Salmonella typhimurium* and *E. coli* F4 was determined with a quantitative microbiological microplate-based assay by measuring the optical density as growth parameter of adhering bacteria. The YD was suspended in phosphate buffered saline (0.01%), aliquots of 100 µL/well were pipetted in a 96-well plate and incubated for 16-18 hours at 4°C. With the microplate washer (Tecan 5082 M8/4R Columbus Plus)/PBSTween20 the plate was washed three times. Subsequently, the bacterial suspension (either *Salmonella typhimurium* or *E. coli* F4) was adjusted to OD 0.01 and added in the wells of the microplate. Bacteria were allowed to adhere for 60 minutes at 37°C and afterwards washed 6 times with microplate washer/PBSTween to remove non-adherent bacteria. The wells were filled with 200 µL of TSB and coated with one drop paraffin-oil. The microplate was placed in a

microplate reader (Tecan Genius), incubated for 18 hours at 37°C and an OD of 690 nm. Data were recorded every 15 minutes (according to Ganner et al., 2010).

Performance and sample collection. At the beginning of the experiment all chicks were weighed individually and then assigned to treatment and replicate. During the experiment chicks were weighed by pen on d 14 and individually on d 35. Feed intake was determined from 1-14 and 14 to 35 d of age. Mortality was determined and recorded twice daily. An independent licensed veterinarian checked all chicks on a regular basis. On d 35 one bird from each pen was randomly selected and euthanized by CO₂ asphyxiation and the jejunum (distal duodenum to meckles diverticulum) was gently folded in half length-wise and a 5 cm portion at mid-length removed. Following excision, the segments of jejunum were flushed with 10% neutral buffered formalin (NBF) and placed in a solution of 10% NBF.

Blood samples. Blood samples were collected from the wing vein from one chicken per pen (8 chickens per treatment) into EDTA tubes on day 35. The tubes of blood were sent to a clinical laboratory (LABOKLIN GMBH & CO.KG, Linz, Austria) and analysed by CELL-DYN 3500 (Abbott Laboratories, IL, USA) for the following: leukocytes (g/l); heterophils (% of leukocytes), lymphocytes (% of leukocytes) and monocytes (% of leukocytes). The CELL-DYN® 3500 is a multi-parameter flow cytometer which counts and differentiates white blood cells (WBC) based on the principle of multi-angle polarized scatter separation (M.A.P.S.S.). The WBC count is determined by both, optical and impedance methods.

Histology. Jejunal sections were processed and embedded in paraffin blocks, and two slides were prepared from every tissue block; one for periodic acid Schiff (PAS) reagent staining (Armed Forces Institute of Pathology, 1992), and one for TUNEL DNA fragmentation staining. From different locations on the jejuna segment three sections (3µm) were placed on every slide for histological staining. For the PAS staining slides were

counterstained with Harris Hematoxylin solution (catalog no. 1092530500, Merck KGaA, Darmstadt, Germany), dehydrated and mounted with Entellan (catalog no. 107961, Merck KGaA, Darmstadt, Germany). A modified light microscope with an ocular micrometer (MXK23003, Nikon GmbH, Vienna, Austria) was used to measure the length of twelve villi per bird, and verified via a reference micrometer (LA_CALSLI, Nikon GmbH, Vienna, Austria). The number of apoptotic enterocytes was determined with the DeadEnd™ TUNEL assay kit (cat. No. G7130, Promega, Madison, WI, USA). The TUNEL staining is a method for detecting DNA fragmentation resulting from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA, which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs, which have been secondarily labeled with a marker. The histological slides were counterstained with Methyl green. Nine villi per bird were enumerated for calculating the number of apoptotic cells per villus.

Statistical analysis. The data were analyzed using the MIXED procedure of SAS® version 9.1 (SAS Institute, Inc., Cary, NC). Differences between means were determined by the PDIFF comparisons. Statements of significance were $P < 0.05$ unless noted otherwise.

Results

Yeast derivative. The mannan-, glucan-, and protein contents of the YD used in this experiment were 17, 25, and 43%, respectively. Thus, calculated content of mannan and glucan in the 0.1 and 0.2 kg/t were 0.017%, 0.025% and 0.034%, 0.05%, respectively. In the *in vitro* binding assay, the YD bound *Salmonella typhimurium* at 5×10^5 CFU/mg and *E. coli* F4 with 9×10^3 CFU/mg.

Performance. On d 35 the birds fed the 0.1% YD were significantly heavier (1520 g) compared to those fed 0.2% YD (1391 g) and the control group (1374 g; $P=0.05$; Table 2). The BW at 14 d of age was not affected by diet ($P>0.05$). Between d 14-35, birds fed with 0.1% YD had significantly increased BW gain compared to the control group and the birds fed 0.2% YD ($P=0.05$), thereby influencing BW gain from d 1 to 35 for the birds fed the 0.1% YD ($P<0.05$). The feed intake was not influenced by the treatments ($P>0.05$; Table 3). The feed-to-gain ratio (FCR) from d 14 to 35 was significantly decreased in birds fed 0.1% (1.861) and 0.2% (1.815) YD compared to the control group (2.156, $P<0.05$). There was no significant difference of the FCR from d 1-14 ($P>0.05$), but between d 1-35 there was a difference between the 0.2% yeast group (1.878) and the control (2.125) as well as a trend towards significance ($P=0.061$) between the control and birds fed the 0.1% YD. Mortality was not significantly influenced by the treatments ($P>0.05$) from day 1 to 35 (data not shown).

Blood parameters. There was no significant difference on the number of leukocytes, and also no significant difference on the ratio of heterophiles, lymphocytes and monocytes ($P>0.05$) between control and YD groups (Table 4). Similarly, there was also no significant difference in heterophil/ lymphocyte ratios ($P>0.05$).

Jejunum Morphology. There was no significant difference in villus length, crypt depth or total goblet cell number per villus (Table 5). However, birds fed with 0.1% and 0.2% YD had a significant higher density of goblet cells than the control group ($P<0.05$). The number of apoptotic enterocytes in the jejunum of birds fed 0.1% and 0.2% YD groups was significantly ($P<0.05$) lower than in the control group.

Discussion

The aim of present study was to evaluate the influence of two different concentrations of a YD on performance, blood profile and gut histology of broilers. The results showed that especially the lower (0.1%) concentration of the YD had a positive influence on the performance parameters of the broiler. Birds fed with 0.1 % YD had 10.6 % higher BW on d 35 compared to the control group ($P=0.05$).

Some controversial results concerning the health effect of yeast derivatives are notable comparing the literature and the results of present study. Inasmuch, several variables need to be considered before adequate comparisons can be made, including: origins of the yeast derivatives, yeast strain, source of the yeast strain, production method, mannan and glucan content. A weak point in the interpretation and comparison of literature data is the lack of information given on the characteristics of the yeast products used including the chemical characterization which is missing in most publications. Furthermore, some of the published studies have been conducted either with MOS, yeast extract or yeast culture, which could plausibly be associated with different key physiological and/or immunological modes of action. Therefore it is important to exactly define the characteristics (classification of yeast product and composition) of the yeast product.

In terms of growth performance Zhang et al. (2005) also reported a significant increase in BW gain (1623 g/bird) when fed 0.3% yeast cell wall compared to a negative control group with a BW gain of 1550 g/bird from week 0 to 5, however there was no significant difference in feed intake ($P>0.05$). Similarly, Gao et al. (2008) reported that the supplementation of 2.5 g/kg yeast culture significantly improved BW (2459 g) of 42 d-old broilers compared to a control group (2378 g). Shareef and Al-Dabbagh (2009) reported a significant increase of BW gain from d 1 to 21 in broiler chicks when baker's yeast was added at a rate of 1 (309.6

g), 1.5 (318.3 g) and 2% (323.9 g) to the diet compared with the control (201 g). Savage and Zakrzewska (1996) investigated turkeys supplemented with yeast derived mannanoligosaccharides (0.1%) and noted they had significantly ($P<0.05$) increased BW gain (2531g) from week 4 to 8 compared to the control group (2202 g). Other studies (Hayat et al., 1993; Bradley and Savage, 1995; Savage and Zakrzewska, 1995) also showed improved performance (weight gain and final weight) in turkeys supplemented with yeast products (either mannanoligosaccharides or yeast culture).

Yeast derivatives have been described to positively affect health and performance parameters of other animal species such as swine. Shen et al. (2009) reported an increased BW gain in a yeast culture (5g/kg) group compared to a control group in nursery pigs together with a greater ($P<0.05$) digestibility of dry matter, crude protein, gross energy, and jejunal villus length and villus height: crypt depth ratio ($P<0.05$). Similarly, van der Peet-Schwering et al. (2007) reported an significant ($P=0.05$) increase of BW in weaning pigs on d 35, the animals fed with 0.125% yeast culture had 23.3 kg and the pigs fed 0.125% yeast culture and 0.2% yeast cell wall had 23.6 kg in comparison to the control group with a BW of 22.7 kg.

Although in some studies (Benites et al., 2008; Baurhoo et al., 2007) supplementation of a mannanoligosaccharide product derived from yeast did not significantly ($P>0.05$) improve FCR, in the present study, however, there was a positive influence on FCR from day 14 to 35, the 0.1% YD group decreased the FCR from 2.156 to 1.861 and the 0.2% YD reduced even further to 1.815.

Gao et al. (2008) reported a significant improvement in FCR from d 1 to 42 in a 2.5 g/kg yeast culture supplemented group (1.95) compared to a negative control group (2.03). Similarly to Gao et al. (2008), the present trial demonstrated no significant difference in the FCR between d 1 and 14, but the YD did improve the FCR between d 14 and 35 ($P<0.05$). Similarly, Satin et al. (2001) reported an improved BW gain of 4.3% from day 1-21, and a

decreased FCR of 7.95% from day 1-21 in broiler supplemented with 0.2% *Saccharomyces* cell walls compared to a non-supplemented control group. Also in a study with turkeys (Sims et al., 2004) a 6.25% improved FCR was observed in a group supplemented with a yeast cell wall product compared to a control group at week 15.

No significant differences in mortality were noted between the three groups which is in agreement with other studies where yeast derivatives did not show any significant difference on mortality in broilers (Benites et al., 2008; Gao et al., 2008) and in turkeys (Sims et al., 2004; Solis de los Santos et al., 2007). The inclusion rate and thus supplemental concentration of mannan, glucan, and/or the combination therein appears to be a very important point according to performance data in broilers. Similarly to the present study, Gao et al. (2008) reported that the lower inclusion rate (2.5 g/kg) of a yeast culture significantly increased the BW ($P<0.05$) on day 42 in broiler, but the two higher (5 and 7.5 g/kg) concentrations did not significantly increase the BW compared to a control group. Also the average daily gain (d 1 to 42) and the FCR (d 1 to 42) was only ($P<0.05$) improved significantly by the 2.5 g/kg inclusion level compared to the control group in this study. So it seems like there is an optimal dosage, which is the most effective and higher concentration can reverse the positive effect of the yeast supplementation. If there is a high pathogenic load higher inclusion rates of the product might work better since more of the pathogenic bacteria can be bound; however if there is a lower pathogenic load lower inclusion rates of the product might be a more effective dosage. The present study was conducted under relatively clean experimental conditions. As already mentioned, chemical characteristic of many yeast products is missing in literature and therefore adequate and sound comparisons cannot adequately be made.

The capacity of the YD, fed in the present study, to bind *in vitro* *Salmonella typhimurium* and *E. coli* F4 was 5×10^5 CFU/mg and 9×10^3 CFU/mg, respectively. *In vitro* data (Ganner et

al., 2008, unpublished data) indicate that *Salmonella* and *E. coli* binding are due in part to the mannan content of the yeast. Whether this binding reduced bacteria and/or pathogens in this study is uncertain. Nevertheless, bird performance was enhanced.

The present study showed no significant effect on the intestinal morphology concerning the villus length and crypt depth, although there were positive effects on the body weight gain and FCR. Another study also reported no significant difference in intestinal villus length and crypt depth (Baurhoo et al., 2009); unfortunately no performance data were evaluated. On the other hand, Zhang et al. (2005) reported a significant ($P<0.05$) increase in villus length in a yeast cell wall group (437 μm) in comparison to a control group (397 μm) in 21 day-old broiler; however no difference in crypt depth. Also a growth promoting effect could be observed with a significant ($P<0.05$) improved BW gain, which might be explained by increased villus length. Satin et al. (2001) made a similar observation with a 0.2% yeast cell wall supplemented group, where the BW gain was improved together with increased villus length and decreased crypt depth at day 7, but at day 28 and 35 there was no significant difference between the trial groups. Villus length can be an indicator of the absorptive surface, which is available to the animal. Shortening of the villi means a decrease of absorptive capacity. Crypt depth can be closely related to the tissue turnover, so deeper crypts can be a sign of an increased turnover (Yason et al., 1987), as the crypt region is usually considered as the site for cellular proliferation. In broilers and turkey poults, however, cellular proliferation is not confined to the crypt and can occur along the length of the villus (Uni et al., 1998; Applegate et al., 1999).

No effect on the total number of goblet cells could be observed, however a significant effect on the goblet cells density was notable. In the birds fed the 0.1% YD the goblet cell density was 41.16% higher and in the birds fed 0.2% YD was 28.7% higher compared to the control group ($P=0.005$).

Baurhoo et al. (2009) reported that a yeast cell wall product (0.15% starter diet; 0.1% grower diet) significantly increased the total number of goblet cells in 24 (174.2) and 34 day (196.5) old broiler in comparison to a negative control group (135.59; 171.26) and a virginiamycin antibiotic group (127.54; 140.10). There are several other studies which could also observe similar results in turkeys (Baurhoo et al., 2007; Soils de los Santos et al., 2007). The intestinal mucin is very important because it is a first line of host defense against invading pathogens. Thus increased mucin production can be a great advantage for the animal due to a greater elimination of intestinal pathogens and therefore an improved protection system against intestinal infections. In the present study the number of apoptotic cells was 27.4% (0.1% YD) and 38.4% (0.2% YD) lower compared to the control group ($P=0.02$). The GIT responds to stress (pathogens, anti-nutrients, etc.) by increasing enterocyte migration, to remove damaged enterocytes, and/or to increase the rate of apoptosis (Applegate, 2009). When enterocytes act in this manner, they cannot fully differentiate, so undigested disaccharides can enter into the lower digestive tract causing microbial growth and subsequent osmotic and secretory diarrhea (Zijlstra et al., 1997). Differences in enterocyte turnover, however cannot directly be ascertained in the current study. Nevertheless, the lower number of the apoptotic enterocytes in the birds supplemented with 0.1% yeast may correlate with subsequent improved performance

No statistical differences in the blood cell counts could be observed ($P>0.05$). Numerous studies, summarized by Kogan and Kocher (2007) indicate that yeast derivatives, such as β -glucan fractions and mannan-oligosaccharides modulate parts of the immune system and enhance the functional status of macrophages. However, little data have been published regarding the effect of yeast derivatives on the number of white blood cells. Contrary to the present data, Huff et al. (2007) reported elevated percentage of heterophils in peripheral blood by yeast extract supplemented (inclusion rate: 0.05% and 0.1%) diets in turkeys

($P=0.02$). Cetin et al. (2005) investigated hematological and immunological parameters in turkeys fed with a mannanoligosaccharide preparation (0.1%). Contradictions within the literature, however, may be more related to compositional differences between yeast, which in earlier studies, were not analyzed.

Conclusion

In conclusion, the YD when fed at 0.1% of the diet (0.017% and 0.025% of mannan and glucan, respectively) influenced positively the final BW, the daily BW gain, FCR, and jejunal goblet cell density and reduced the number of apoptotic enterocytes. Both concentrations (0.1%, 0.2%) of the YD had positive influences on performance and gut morphology, and the 0.1% inclusion rate seemed to be the optimal concentration. Increased goblet cell density as well as the pathogen binding capacity (characterized *in vitro*: *Salmonella typhimurium* 5×10^5 CFU/mg; *E. coli* F4 9×10^3 CFU/mg) might have protected the birds against primary infections and could have been reasons for the improved performance.

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Table captions:

Table 1: Basal diet formulation of the starter and grower diet

Table 2: Effect of yeast derivative to broiler diets on body weight (BW) and BW gain

Table 3: Effect of yeast derivative supplementation to broiler diets on feed intake and feed-to-gain (FCR)

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Table 5: Effects of yeast addition to broiler diets on jejunum morphology at 35 days of age

Table 1: Basal diet formulation of the starter and grower diet

Ingredients %	Diets	
<i>Raw materials, g/kg</i>	Starter diet (0 to 14 d of age)	Grower diet (14 to 35 d of age)
Corn (maize)	579.3	597.5
Soybean meal, 48% CP	312.5	296.0
Vitamin-mineral premix	62.5 ^a	60.0 ^b
Soy oil	25.0	20.0
Vegetable oil blend	12.5	25.0
L-Lysine	3.8	1.5
Monocalcium phosphate	2.5	-----
L-Threonine	1.3	-----
DL-Methionine	0.8	-----
<i>Nutrient composition</i>		
ME _n , kcal/kg	3021	3095
Crude protein, g/kg	208.1	197.4
Methionine, g/kg	5.49	4.59
TSAA, g/kg	9.02	8.02
Lysine, g/kg	14.06	11.78
Threonine, g/kg	9.27	7.77
Calcium, g/kg	11.96	11.10
Phosphorus, g/kg	8.47	7.68

^aSupplied per kg of feed: vitamin A 14,450 I.U.; vitamin D₃, 5,000 I.U.; vitamin E, 100 mg; vitamin K₃, 3.6 mg; vitamin C, 80 mg; vitamin B₁, 3 mg; vitamin B₂, 6 mg; vitamin B₆, 6 mg; vitamin B₁₂, 40 mcg; nicotinic acid, 90 mg; Ca-pantothenic acid, 16 mg; cholinechlorid, 525 mg; folic acid, 2 mg; biotin, 270 mcg; copper, 25 mg; zinc 75 mg; iron 125 mg; manganese, 75 mg; cobalt, 1.25 mg; iodine, 2.5 mg; selenium, 0.5 mg

^bSupplied per kg of feed: vitamin A 13,850 I.U.; vitamin D₃, 4,800 I.U.; vitamin E, 95 mg; vitamin K₃, 3.4 mg; vitamin C, 80 mg; vitamin B₁, 3 mg; vitamin B₂, 8.5 mg; vitamin B₆, 6 mg; vitamin B₁₂, 40 mcg; nicotinic acid, 85 mg; Ca-pantothenic acid, 15 mg; cholinechlorid, 500 mg; folic acid, 2 mg; biotin, 260 mcg; copper, 24 mg; zinc 72 mg; iron 120 mg; manganese, 72 mg; cobalt, 1.2 mg; iodine, 2.4 mg; selenium, 0.5 mg

Table 2: Effect of yeast derivative to broiler diets on body weight (BW) and BW gain

Diet	Days of age					
	1	14	35	1-14	14-35	1-35
	----- (BW, g/bird) -----			----- (BW gain, g/bird/d) -----		
Control	37.7 ¹	320.6	1374 ^b	20.21	50.15 ^b	38.18 ^b
0.1% yeast	37.4	315.8	1520 ^a	19.88	57.35 ^a	42.37 ^a
0.2% yeast	37.3	305.3	1391 ^b	19.14	51.73 ^b	38.69 ^b
SEM	0.25	11.9	44.6	0.86	1.76	1.28
Probability of diet effect	0.43	0.60	0.05	0.62	0.017	0.05

^{ab} Means in columns with no common superscripts differ significantly ($P \leq 0.05$).

¹ Means represent 8 pens per diet, 26 birds per pen.

Table 3: Effect of yeast derivative supplementation to broiler diets on feed intake and feed-to-gain (FCR)

Diet	Days of age					
	1-14	14-35	1-35	1-14	14-35	1-35
	----- (feed intake, g / bird) -----			----- (FCR) -----		
Control	571.5	2218	2790	2.019	2.156 ^a	2.125 ^a
0.1% yeast	609.0	2252	2862	2.169	1.861 ^b	1.919 ^{ab}
0.2% yeast	569.1	1977	2546	2.129	1.815 ^b	1.878 ^b
SEM	36.1	112.7	138.7	0.093	0.079	0.074
Probability of diet effect	0.69	0.19	0.26	0.51	0.012	0.061

^{ab} Means in columns with no common superscripts differ significantly ($P \leq 0.05$).

¹ Means represent 8 pens per diet, 26 birds per pen.

Table 4: Effect of yeast addition to broiler diets on blood cell profiles at 35 days of age

Diet	Leukocytes	Monocytes	Heterophils	Lymphocytes	Heterophil:Lymphocyte
		----- (G/L) -----			
Control	12.67	1.047	6.88	4.33	1.587
0.1% yeast	13.07	1.745	7.88	3.48	2.769
0.2% yeast	12.85	1.651	7.60	3.49	2.375
SEM	1.61	0.53	1.20	0.57	0.436
Probability of diet effect	0.98	0.61	0.83	0.49	0.17

^{ab} Means in columns with no common superscripts differ significantly ($P \leq 0.05$).

¹ Means represent 8 pens per diet, 1 bird per pen.

Table 5: Effects of yeast addition to broiler diets on jejunum morphology at 35 days of age

Diet	Villus height	Crypt depth	-----Goblet cells-----		Apoptotic enterocytes
	----- (µm) -----		(# / villus)	(# / 10 µm villus)	(# / villus)
Control	1006	89.4	108.3	1.069 ^b	9.46 ^b
0.1% yeast	921	82.1	137.6	1.509 ^a	6.87 ^a
0.2% yeast	931	88.1	128.2	1.376 ^a	5.83 ^a
SEM	46.9	4.8	10.4	0.094	0.95
Probability of diet effect	0.33	0.51	0.12	0.005	0.02

^{ab} Means in columns with no common superscripts differ significantly ($P \leq 0.05$).

¹ Means represent 8 pens per diet, 1 bird per pen.

Probiotics and phytogenics for poultry: Myth or reality?

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Probiotics and phytogenics for poultry: Myth or reality?¹

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Primary Audience: Nutritionists, Researchers, Veterinarians

SUMMARY

Removal and restriction of subtherapeutic antibiotics from poultry diets in many parts of the world has amplified interest in improving intestinal health and nutrient utilization. Some probiotic (direct-fed microbials) and plant-derived (phytogenic) feed additives are gaining market presence. Defined probiotic cultures have the potential to succeed, in large part because of in vitro screening and selection. However, regulatory approval delays, particularly in Europe, have stymied the commercial application of some microorganisms in poultry diets. Phytogenic feed additives have demonstrated ranges of antimicrobial activities in vitro and are building a track record of improvements in bird performance. Hesitation by nutritionists to incorporate these feed additives are due in part to 1) unfamiliarity, 2) the overselling of plausible effects by industry, 3) product inconsistency, 4) a lack of documented physiological and microbiological effects in vivo, and, in the case of probiotics, 5) a lack of documentation of persistence.

Key words: competitive exclusion, essential oil, feed additive, phytogenic extract, poultry, probiotic

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DESCRIPTION OF PROBLEM

The poultry industry is inundated with numerous options when it comes to feed additives purported to aid in performance, alleviate symptoms associated with a particular insult, or both. Many of these products have been grouped as antibiotic growth-promoting (AGP) replacements, but all fall short of specific physiological effects elicited by the AGP themselves. These

products are diverse and include enzymes, vitamin metabolites, plant extracts, competitive exclusion (CE) products, probiotics, prebiotics, yeast components, organic acids, short- and medium-chain fatty acids, bacteriocins, bacteriophages, and antimicrobial peptides, to name a few.

In the past, AGP replacements were the gold standard by which growth promotion and

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disease resistance were measured. Used since the 1940s, subtherapeutic doses of antibiotics have enhanced bird performance by increasing growth, improving FE, favorably altering intestinal bacteria, and reducing the incidence of disease. The exact mechanisms by which these improvements occur, however, are still not fully understood. Currently, 4 mechanisms of growth promotion have been proposed by various scientists. Because researchers have indicated that orally dosed antibiotics do not promote growth in germ-free chicks [1], each of these proposed mechanisms is based on the hypothesis that the presence of bacteria in the intestine reduces bird growth, and includes hypotheses that 1) antibiotics inhibit the occurrence of subclinical infections, 2) antibiotics reduce the production of growth-depressing microbial metabolites, 3) antibiotics reduce the use of nutrients by intestinal microbes, and 4) antibiotics allow for enhanced uptake of nutrients because they have been shown to reduce the thickness of the intestinal wall [2–4]. Regardless of the fact that the exact mechanisms of antibiotic-mediated growth promotion are currently incompletely understood, most researchers support the theory that antibiotics reduce the overall numbers of gut bacteria, which may promote growth [5]. In addition, direct-fed antibiotics are not efficacious in all situations; rather, the improvement in feed conversion is more pronounced in unsanitary environments [1]. This is due in part to an intestinal response to the presence of more bacteria and the production of inflammatory cytokines, thereby eliciting an acute phase response and the shunting of nutrients toward support of the immune system [6].

When it comes to interest in products of this nature, often researchers, nutritionists, and veterinarians will make direct comparisons with characteristic benefits of AGP, namely, improvements in feed conversion attributable to their antimicrobial activity in the digestive tract. Although no singular in-feed replacement exists at present to elicit a range of similar responses, the AGP-replacement products do have documented attributes, including, but not limited to, performance improvement, immunomodulation, improvement of in-feed hygiene, bacteriostatic effects on gram-positive or gram-negative flora, and reduction of food-borne pathogens.

In several cases, these products have a reasonable track record in the field for improvements in feed conversion, but information is lacking on their physiological, immunological, and *in vivo* microbiological modes of action. Thus, the end user is often at a loss regarding which product(s) to use and when to use them. Accordingly, much of the mode(s) of action for many of these products for poultry are based on *in vitro* evidence or studies conducted with humans, rats, and other species.

However, specific properties of these classes of additives have been demonstrated, ranging from pathogen exclusion by probiotic bacteria to feed intake responses to essential oils. The focus of this review is not to reiterate the range of *in vivo* effects noted within the literature, but rather to point to specific examples of where and how 2 particular classes of compounds have been developed, namely, probiotics (including CE products) and phytogetic plant extracts. Further, limitations to their application and development are discussed.

PROBIOTICS: PRODUCT DEVELOPMENT AND APPLICATION LIMITATIONS

Several criteria should be considered in the development and use of an ideal CE or probiotic product. Selection of probiotic strains should consider attributes, including being from the species it is being applied to, nonpathogenic, technologically suitable for industrial processes, acid- and bile-resistant, able to produce antimicrobial substances, able to modulate immune responses, and able to influence the metabolic activities of the gut [7]. No single strain, however, is likely to completely fulfill all these criteria. Therefore, in practice, the choice of an economically feasible probiotic is always a compromise between microbiological, technological, performance-promoting, and registration capabilities of the strains tested.

A major point for developing new strategies is to better understand the mechanisms through which the probiotic organisms could protect the intestinal environment from damage induced by pathogens. The intestine is a complex system in which a continuous dialog between the mucosal barrier, microflora, and local immune system

occurs, and nutritional modifiers are likely to interact with any of these constituents [8, 9]. Thus, better insight into how probiotics and CE products work is important to understand their role in intestinal cell protection and to select more efficacious strains.

Probiotics: Product Development and Purported Benefits

Probiotic Selection. Probiotics have gained popularity as “functional” supplements for food (humans) and feed (livestock and poultry). The most commonly used probiotics are strains of lactic acid bacteria (stemming from use in dairy products) and bifidobacteria (stemming from higher proportions in breast milk-fed infants vs. bovine milk-fed infants) [10, 11]. Other organisms also used as probiotics include *Bacillus* spp., *Bacteroides* spp., *Escherichia coli*, *Propionibacterium* spp., *Streptococcus* spp., yeasts, and various fungi [12]. Although, in human and poultry nutrition, the concept of probiotics seems to be comparable, the requirements for their application in feed differ considerably from those in human food. In contrast to the long-term effects expected by the human consumer, microorganisms used as feed additives are designed to produce a quick response, especially at times of need (e.g., postnatal stage, weaning), and are present in a larger quantity of feed; therefore, they must survive the rigors of feed processing and storage.

Similar to human probiotic development, protection against diarrheal diseases and modulation of the immune system have been established as the main goals of probiotic use. Survival in the gastrointestinal tract (GIT; i.e., resistance to gastric acid and bile acids) and adherence to mucosal cells are considered to be important selection criteria for probiotic activity in humans [13]. Ingestion of probiotic lactobacilli has been shown to reduce the duration of several types of diarrheal diseases, including those induced by rotavirus infection, enteropathogenic *E. coli*, *Salmonella enterica*, *Shigella flexneri*, and *Helicobacter pylori* gastroenteritis [14, 15]. Some probiotic preparations have been examined for their effectiveness in the prevention and treatment of antibiotic-associated diarrhea and inflammatory bowel disease [16, 17].

For all animals, the microbial colonization of the GIT at a young age is a critical time period. Commercially produced poultry lack the natural contact between chicks and mother hens, resulting in a delayed development of the intestinal microflora. As a consequence, day-old chicks that do not establish protective microflora immediately after hatching are susceptible to pathogen colonization (especially *Salmonella* and *Campylobacter*) [18, 19].

One of the main strategies of probiotic product development relies on the benefit from the competitive nature of intestinal bacteria to exclude pathogens that negatively affect bird performance or food safety, a phenomenon called CE. The pioneering evidence of the CE concept, which involved feeding fecal contents from an adult bird to day-old chicks, was originally designed for *Salmonella* reduction in chickens [20]. However, it has been expanded to protecting against enteropathogens such as enterotoxigenic *E. coli*, *Clostridium perfringens*, *Listeria*, and *Campylobacter* spp. [21]. The symbiotic microbes of the gastrointestinal environment are thought to enhance resistance to infection by competing with pathogens for nutrients or attachment sites, or more directly by antagonistic action against undesirable microorganisms (i.e., a barrier effect) [21].

The subject of CE has been extensively reviewed [21, 22]. Most effective commercial CE products, however, include undefined (e.g., freeze-dried intestinal material or cultures) or partly defined microbial cultures derived from the intestinal contents or mucosa. Use of these undefined or partially defined cultures is a very simple approach, but in fact, in many countries it is impossible to register such an undefined feed supplement for placement on the market. In Europe, for example, only well-defined microbial feed additives are accepted by legislation [23–25]. Although the probiotic concept is theoretically a sound approach for supporting bird performance and health without the side effects of antibiotics, it is not simple to select and introduce the optimal strains at the optimal conditions (e.g., time) to the bird in an efficient way. However, if the natural microflora is to serve as a source for strain selection, there are many possibilities to choose from. Of the present strategies, multispecies or strain combinations of gut

bacteria might provide several advantages compared with the use of single strains, simply by an additive (complementing) or synergistic effect [26]. Several authors have suggested that multiple strains may be more useful than a single strain because they may act at different sites, in various modes, and probably in a synergistic manner [7, 27, 28]. It has also been hypothesized that mixtures of obligate and facultative anaerobic gut bacteria are more effective because the facultative anaerobes reduce oxygen levels in the anaerobic regions of the GIT, permitting the establishment of strictly anaerobic bacteria [29–33].

In the European Union, single-strain preparations dominate the market. To date, only 12 species are authorized as feed additives: *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus farciminis*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus infantarius*, and *Saccharomyces cerevisiae* [34]. In non-European and non-US markets, in contrast, mostly complex cultures with an undefined composition (enriched from the intestinal microflora of healthy animals) or preparations consisting of a multitude of enteric bacteria are used for CE [35, 36]. Accordingly, current knowledge of synergism of strains within CE cultures as well as the effective species of the various bacterial genera is still inadequate [37].

In view of the risks associated with the total European ban on antibiotic growth promoters (e.g., increased use of therapeutic antimicrobials), several European Union projects (e.g., C-EX, PoultryFlorGut, REPLACE, PROPATH) have been initiated to find alternative ways of preventing and treating animal infections that are also a risk to humans. The C-EX project, titled “Development of a Competitive Exclusion Product for Poultry Meeting the Regulatory Requirements for Registration in the European Union,” for example, had the aim of establishing an adequate combination of probiotic strains for their combined use in chickens. The end result of this project was a feed additive containing 5 strains, using various bacterial species derived from different niches from the gut of chickens, which, in contrast to undefined products, should meet the requirements for registration in the Eu-

ropean Union [28]. The strains were selected from 477 well-characterized strains originally isolated from the crop, jejunum, ileum, and ceca of the gut of chickens, thus providing a rationale for their safe and efficacious use as in-feed additives for chickens [38].

Regulatory Hurdles and Lessons Learned.

One of the hurdles restricting the breadth, number, and speed of approval of products of this nature is the regulatory and registration process, the scope of which varies from country to country. Usually, the European Union and the United States are considered the more stringent, and registration dossier components in other countries use these as models.

In the United States, there is a well-functioning regulatory system for microbial cultures for food and feed, which is based on the GRAS (Generally Recognized As Safe) notification scheme and which encourages industry to be more open about the microbial applications in the food sector [39]. In practice, GRAS status can be achieved either by an established history of safe use of the microorganism in food (dating before 1958) or by a positive safety evaluation by qualified, independent experts. In the European Union, microorganisms used as feed additives are comprehensively regulated [34], whereas in the past, the use of conventional probiotic organisms in various human applications were not particularly regulated [38]. This has led to the illogical situation in which the same strains used freely in human foods have been the subject of stringent safety assessments when seeking approval as feed additives. Safety aspects take up most of the application dossier, with the intention of ensuring that microbial feed additives are innocuous to target animals, users, and consumers [40].

With some probiotic strains, the biosafety is less clear. For example, enterococcal strains have been used positively as probiotics or in the production of certain cheeses, yet other enterococcal strains have been isolated from clinical infections and have been reported to be involved in septicemia [41]. Therefore, in Europe the “safe use” status of newly isolated organisms had to be confirmed by a broad range of safety studies with respect to the target species (tolerance test, effect on the microflora), the worker (skin and eye irritancy, skin sensitization, toxic

effects on the respiratory system, systemic toxicity), the consumer (2 different genotoxicity tests, 2 mutagenicity tests, a 90-d oral toxicity study), and the environment (if the organism was not of gut origin and not already ubiquitous in the environment) before being incorporated into feed. This safety testing equates microorganisms with chemical substances, often making it difficult for both the authorities and the applicant to apply these studies to microbes. Because of these very strict regulations, only a few feed additives containing generally only 1 or, in some exceptions, 2 strains are currently available in Europe. Very recently, the Qualified Presumption of Safety (QPS) approach, similar in concept and purpose to the GRAS definition used in the United States, has been introduced and applied to a selected group of microbial species, allowing strains belonging to species falling within a QPS group to enter the market without the extensive toxicity testing [23–25]. Microorganisms not considered suitable for QPS (or where there is no sufficient body of information indicating that all strains of the species can be presumed to be safe) remain subject to a full safety assessment [42]. Particular attention is focused on the presence of transmissible antibiotic resistance and on the risk for production of harmful metabolites. Evaluating the strains for their antibiotic resistance profile and the transferability of any resistance is crucial for all strains. The evaluation begins with an assessment of the minimum inhibitory concentration by a wide range of antibiotics. If the strain is proved to be resistant to a specific antibiotic (i.e., when the minimum inhibitory concentration exceeds the threshold defined by the European Food Safety Authority), the genetic basis of the resistance has to be determined. Only if the resistance is proved to be intrinsic or acquired through a genomic mutation is the strain acceptable.

The future of probiotic feed additives will very much depend on the regulatory developments in the area. The stringency of requirements for proving quality, efficacy, and especially safety of probiotic preparations for livestock and poultry will naturally determine the opportunity to bring new products on the market. Currently, the success of these products is limited because of the various requirements for the registration process, and innovation of non-QPS strain registra-

tion may be stymied because of the additional time and expense of safety testing.

In Vitro Screening. Despite the difficulties of applying the results obtained by in vitro methods to the in vivo situation, the initial screening of strains remains a useful first step in detecting probiotic candidates. Different strains display distinct features, some of them showing strong antagonistic activities, others having advantageous technological features (e.g., ease of production or resistance to thermal processing), and others, in turn, scoring in important safety aspects (e.g., antibiotic susceptibility patterns). Therefore, it is more likely that beneficial effects in the complex environment of the gut will require the introduction of a mixture of strains.

In vitro screening techniques for probiotic candidates that have pathogen exclusion capabilities have been well documented. For example, Bielke et al. [43] described an in vitro screening method for probiotic candidates that resulted in a defined culture of 24 selected isolates that were effective against *Salmonella* in young poult. Screening for multiple positive traits of probiotic candidates has also been documented. For example, in the European Union C-EX project, a variety of intestinal bacteria (with special focus on lactic acid bacteria and bifidobacteria) were isolated from various chicken GIT sources and were assayed for antimicrobial activity, adherence properties, fermentation characteristics, and antibiotic susceptibility patterns [28]. Out of 121 well-characterized strains, a reduced number of 90 stains exhibited the ability to inhibit a pathogenic indicator strain of *S. enterica* serovar Enteritidis. Antagonism to a series of pathogenic strains affiliated with *S. enterica* serovar Choleraesuis, *E. coli* serotypes O157:H7 and O147:H19, *Campylobacter jejuni*, and *C. perfringens* was shown by 20 strains. As indicated by both Giemsa staining and agar plating assays, the ability to adhere to the Caco-2 cells varied considerably among the test strains and was found to be highly strain specific. Five effective strains (*P. acidilactici*, *E. faecium*, *Bifidobacterium animalis* ssp. *animalis*, *Lactobacillus reuteri*, *Lactobacillus salivarius* ssp. *salivarius*) were found to perform consistently well against a broad range of common poultry pathogens and were evaluated with regard to further selection criteria, including growth and fermentation

performance, pH reduction, and biosafety (e.g., lack of virulence determinants, antibiotic susceptibility, and lack of plasmid-linked resistance to clinically relevant antibiotics) [28].

In Vivo Efficacy. Probiotic supplementation has been shown to improve the performance and efficiency of nutrient use in broilers [38, 44–48] and in laying hens and pullets [49–56]. This improvement is presumed to be a result of bacterial antagonism, competition for colonization sites, competition for nutrients, a reduction in the production of toxic compounds, or modulation of the immune system [13]. The end result, in some cases, is improved intestinal health, resulting in greater intestinal enzyme activities and nutrient absorption [49].

Pathogen exclusion by probiotics in poultry has been documented. These include a reduction of *E. coli*, *Salmonella*, *C. jejuni*, and *Emeria acervulina* [57–64]. The question remains whether this is a direct effect or an indirect effect due to immunomodulation. For example, Dalloul et al. [62] noted an earlier production of IFN- γ and IL2, and increased intraepithelial lymphocytes with recognition markers when birds were supplemented with a probiotic compared with unsupplemented birds challenged with *E. acervulina*. When immunosuppressing the birds with a vitamin A deficiency in addition to challenging them with *E. acervulina*, the immunomodulatory effects were confirmed in that feeding probiotics resulted in 4-fold less *E. acervulina* shedding [63].

Technological Limitations. The demonstration of probiotic effects on the level of the host microbiota has been greatly enhanced in recent years by new molecular techniques enabling the detection and identification of microbial species, genera, or groups that are difficult or even impossible to cultivate [65]. However, the exact identification of probiotic organisms on the strain level in fecal or intestinal samples is still a hurdle. Because the probiotic properties seem to be strain specific, every strain claimed to be beneficial for the host should be studied intensively for its properties, both in vitro and in vivo.

For evaluating the efficacy and persistence of an introduced microbial strain, it is necessary to develop monitoring methods that differentiate the nonnative strain from indigenous populations. Various methods have been established

with the aim of specifically tracing industrial or probiotic strains through the GIT. For some strains of lactobacilli and lactococci, labeling with a plasmid-encoded fluorescent protein-encoding gene placed under an inducible promoter has been reported [66]. Site-specific integration of the desired genetic elements into bacterial chromosomes through phage attachment sites has been described for *Lactococcus lactis* [67], *Lactobacillus delbrueckii*, and *L. plantarum* [68]. Insertion of an extra DNA label in a target genome provides a way to monitor the specific strain in various environments. However, the introduction of foreign DNA is generally a scientific approach that is limited by the rather low acceptance among consumers for genetically engineered foodstuffs. Some investigators have circumvented this problem by inserting silent mutations (changes of one or a few bases) in gene coding sequences without affecting the amino acid sequence of the corresponding gene product, but still allowing specific distinction with DNA-based detection [69].

Several currently well-established strain identification and tracking techniques have been applied to probiotic *Lactobacillus* spp. and *Bifidobacterium* spp. These include plasmid profiling [70], ribotyping [71], random amplified polymorphic DNA [72], and DNA fingerprinting of genomic restriction fragments by pulsed-field gel electrophoresis [73, 74]. Such techniques, however, are limited in complex microenvironments such as the gut because they rely on the isolation and cultivation capability of organisms. In recent years, more sophisticated methods have been used for identifying strain-specific sequences in genomes of probiotics for their use as biotracers, with subtractive DNA hybridization methodologies being the most successful in identifying absolute or amplified targets. Promising genome-based tracking techniques, such as suppression subtractive hybridization (SSH), differential display polymerase chain reaction (PCR), representational difference analysis, or microarray [75], allow the identification of specific sequences among highly similar genomes, which, in combination with real-time PCR, can be used in feeding studies to track probiotic strains from the feed through the GIT of the animal. In combination with quantitative PCR, their application will overcome several shortcomings

of conventional approaches (e.g., cultivation dependency, poor discrimination, nonquantitative data). Aside from the search for specific DNA markers for tracking bacteria, SSH as well as its modifications can be further used to study genomic diversity related to exceptional bacterial secondary metabolisms or genes with special microbial functions in the gut [76]. Genes differentially expressed at the mRNA level or genomic differences among microbial strains may be isolated by SSH, making it a useful tool in functional genomic studies.

With the current set of genetic techniques for identification, differentiation, and community characterization, the stage is set for exploring the effects of probiotics or other nutritional modifiers at the bacterial level. Conclusive strain identification and monitoring become vitally important in light of the exploding level of interest, the expanding number of studies, and the major economic investments directed to the broad use of probiotic cultures. The ability to track an industrial strain or group of strains through a feeding trial not only protects proprietary strains, but also allows comparisons between different studies and the unraveling of interactions between bacterial populations, specific organisms, or genes.

PHYTOGENICS AND PLANT-DERIVED COMPOUNDS

Phytogenics, or plant-derived compounds, have been incorporated in livestock and poultry feed to improve productivity. Phytogenics include a broad range of plant materials, most of which have a long history in human nutrition, where they have been used as flavors, food preservatives, and medicines, in solid, dried, and ground forms or as extracts or essential oils [77]. Phytogenic feed additives usually have considerable variation in their chemical composition, depending on their ingredients and the influences of climatic conditions, location, harvest stage, or storage conditions. Hence, differences in efficacy between phytogenic products that are currently available on the market may be attributed mainly to differences in their chemical composition.

Essential oils represent a particular subcategory of phytogenics. They are odoriferous, secondary plant metabolites that contain most of the

active substances of the plant, being mainly hydrocarbons (e.g., terpenes, sesquiterpenes), oxygenated compounds (e.g., alcohols, aldehydes, ketones), and a small percentage of nonvolatile residues (e.g., paraffin, wax) [78]. They are usually obtained from the raw materials through steam distillation. The use of essential oils has a long tradition, for example, in perfumes, food flavors, deodorants, and pharmaceuticals. In human nutrition, their appetizing and digestion-promoting effects have long been recognized [79, 80]. Their chemical composition is highly diverse, including a relatively large number of unidentified substances. Some biologically active compounds found in essential oils of herbs and spices are shown in Table 1 [81].

As shown in Table 1, oregano essential oils contain 14 chemical substances with antioxidant properties. Furthermore, as many as 19 substances have been identified as exerting bactericidal effects. Moreover, oregano is widely available, making it attractive as a feed additive.

The use of synthetic active compounds, such as carvacrol, thymol, limonene, or cinnamaldehyde, is considered an alternative to using natural extracts. Choosing the most suitable combination of ingredients requires extensive research through broad in vitro testing as well as well-designed feeding experiments under standardized conditions.

Effect of Cultivar Type, Growing Conditions, Processing, and Storage on the Active Components or Secondary Plant Metabolites

To guarantee a continuous quality of phytogenic feed additives, strict standardization of the chemical composition in terms of their active ingredients is obligatory. This is not always easy because the levels of active principles in plants or plant extracts may vary considerably, as affected by genetic (i.e., genotype, variety) and external factors (i.e., growing conditions, harvest time, storage, processing). For example, Hüsni Can Baser [82] noted that the oil content of *Origanum vulgare* ssp. *hirtum* harvested in Turkey ranged from 2.3 to 5.4%, whereas the carvacrol content could range from 52 to 61%.

The essential oil content and composition also depend on the part of the plant used to make a phytogenic additive. For example, a study

Table 1. Numbers of different biologically active compounds with different effects present in herbs and spices¹

Herb or spice	Number of identified biologically active compounds				
	Antioxidant	Sedative	Antidepressant	Antiviral	Bactericide
Bay	3	5	—	5	5
Cassia	3	—	—	3	3
Cayenne	9	7	7	6	8
Cumin	5	6	—	7	11
Garlic	9	5	5	5	13
Ginger	6	11	5	6	17
Oregano	14	—	—	11	19
Rosemary	12	6	—	10	19
Sage	7	—	—	—	6
Thyme	4	—	3	3	5

¹Extrapolated from Duke and Beckstrom-Sternberg [81].

comparing the chemical composition of essential oils from buds and leaves of clove showed differences in their main constituents [83]. The differences found between the essential oils of different plant organs can be partly explained by the existence of different secretory structures that are distributed within the plant body [84].

Essential oil production is highly dependent on climatic conditions. In a study investigating the composition of *O. vulgare* ssp. *hirtum* for carvacrol, thymol, γ -terpinene, and *p*-cymene at 23 different localities in Greece, it was shown that the hotter the climate, the higher their total concentration in the essential oil [85]. Moreover, altitude seems to be an important environmental factor [85]. High values of essential oil contents were recorded at low altitudes.

The scents of plants are, in most cases, related to the attraction of pollinators. As such, the emission of volatiles attains its maximum at the time of nectar availability or of pollen maturation, which is when the flower is ready for pollination. Apart from the monthly and annual fluctuations or the changes associated with the vegetative or flowering period of the plant, there are also diurnal fluctuations that seem to be related to the activity of the pollinator [84]. In plants with diurnal pollination, the emission of volatiles attains its maximum during the day, whereas the contrary is observed for those plants having night pollinators (bats, mice, or nocturnal moths). For example, changes in the volatile components emitted from the flowers of honeysuckle throughout 24 h have been shown, wherein the strongest odor was found to be

emitted from 1930 to 0730 h, with its maximum between 1130 to 1530 h [86].

In Vivo Metabolism and Site of Action

As reviewed recently [87], the inclusion of phytochemicals in broiler diets may result in reduced feed consumption at fairly unchanged BW gain, hence resulting in improved feed conversion in the majority of trials reported so far. The exact physiological effects of different active compound(s) in poultry have largely been limited to studies on antimicrobial activity and nutrient digestibility and absorption.

Palatability. Because of their aromatic properties, several phytochemical feed additives have an impact on feed palatability, depending on the applied dosage of the respective ingredients. Their potential to stimulate feed intake, especially in young animals, has been reported in several studies with broiler chicks and weanling pigs [88–90]. However, it may be speculated whether an increase in feed consumption is a consequence of improved digestion rather than enhanced palatability.

Antimicrobial Effects. Numerous plant extracts have shown antimicrobial, anticoccidial, fungicidal, or antioxidant properties [89, 91, 92]. Several in vitro studies have demonstrated a strong inhibition of pathogenic bacteria in the presence of several plant extracts [93]. In vitro studies by Hernández et al. [94] noted that carvacrol, thymol, and cinnamaldehyde inhibited the growth of gram-negative bacteria such as *E. coli* and *S. enterica* serovar Typhimurium. Zrus-

tova et al. [95] reported that in vitro, different essential oils, including lemon myrtle, eucalyptus, and tea tree, strongly inhibited the growth of *C. perfringens*. The antimicrobial action of essential oils has been attributed to their lipophilic character [96], whereby the essential oil(s) may be able to suppress pathogenic bacteria by either penetrating into the cell or disintegrating the bacterial cell membrane.

In vivo antimicrobial effects of different phyto-genic plants in poultry were recently reviewed [97]. Lee and Ahn [98] reported that cinnamaldehyde selectively inhibited *Bacteroides* and *C. perfringens*. Mitsch et al. [99] investigated the effects of essential oils in broilers. A blend of thymol, eugenol, curcumin, and piperin significantly reduced the concentrations of *C. perfringens* in the digesta and feces of the birds, potentially indicating a reduced risk for these birds to develop necrotic enteritis, yet in these field studies, mortality was too low to realize any differences. Reduced levels of *C. perfringens*, *E. coli*, and (numerically) fungi were also obtained in experiments with broilers fed a blend of carvacrol, cinnamaldehyde, and capsaicin [100]. If there is a downside to essential oils, it is that lactobacilli may also be sensitive to the antimicrobial effect of essential oils, as was indicated in the same study [100].

In vivo alleviation of the severity of coccidiosis symptoms has been ascribed to oregano essential oil when fed at 0.3 g/kg [88] in broilers challenged with *Eimeria tenella*. These results were confirmed by Giannenas et al. [101] using ground oregano at a dosage of 10 g/kg. Similar observations were reported with *Artemisia annua* [102], *Sophora flavescens* [103], and *Astragalus membranaceus* [104]. However, a total eradication of *Eimeria* was not obtained in these studies, but rather a lessening of lesion severity and oocyst shedding. Thus, the in vivo effects noted cannot be directly ascribed to direct anticoccidial action without considering the attenuation of intestinal coping mechanisms.

Stimulation of Digestive Enzymes. Some suggest that phyto-genics may stimulate the production of digestive enzymes such as lipase, amylase, or carbohydrases, thus having a beneficial effect on nutrient utilization [105, 106]. However, data regarding this purported mode of action are scarce. Pancreatic lipase and amy-

lase activities were not enhanced by addition of 100 mg/kg of a blend containing carvacrol, cinnamaldehyde, and capsaicin in broilers despite improvements the feed additive elicited on feed conversion [100]. A phyto-genic blend of carvacrol, thymol, curcumin, and piperin in broiler diets had no effect on broiler performance or pancreatic trypsin or α -amylase, or on intestinal maltase or sucrase activity [107]. A subsequent report by the same author noted enhanced activities of pancreatic trypsin and amylase as well as intestinal maltase, but no effect on bird BW or feed conversion when the same phyto-genic feed additive was fed [108]. The question thus remains whether the improved enzyme expression is a direct or indirect effect of the antimicrobial activity.

Gastrointestinal Morphology. A change in morphological parameters, such as villus height, crypt depth, or number of goblet cells, was obtained in several studies when birds were fed diets with supplemental phyto-genics [109, 110]. Jamroz et al. [109] reported that, depending on the type of diet, villus height and crypt depth were affected by dietary supplementation with a phyto-genic feed additive derived from carvacrol, cinnamaldehyde, and capsaicin. When the birds were fed corn-based diets, phyto-genic supplementation significantly reduced crypt depth in the jejunum at 21 d of age. In contrast, the effect was opposite when the birds were fed wheat- and barley-based diets. In the same trial, the blend of phyto-genic ingredients enhanced intestinal mucin secretion and the number of goblet cells on the villi, indicating, in general, a protective effect of these phyto-genic compounds. Because these results are not fully conclusive, there is a need for further investigations into the effect of phyto-genic compounds on gut morphology in poultry.

In Vivo Digestibility. As a result of reduced competition for nutrients between the bird and its gut microflora, as well as a potential stimulation of intestinal enzyme activities and changes in gut morphology, it is assumed that phyto-genics have a positive impact on nutrient digestibility. In a study with broilers, addition of different phyto-genic feed supplements, one based on oregano, cinnamon, and pepper and the other based on sage, thyme, and rosemary, enhanced apparent ileal DM and starch digestibility at 21

d of age [94]. Moreover, phytogenic supplementation increased total tract apparent DM and CP retention. Cobb broilers supplemented with a blend of essential oils derived from oregano, anise, and citrus at 125 mg/kg of diet had increased apparent ileal fat digestibility [111]. In contrast, apparent total tract retention coefficients were not affected when 5 different herbs or essential oils extracted from these herbs were included in broiler diets at a dosage of 10 or 1 g/kg, respectively [112]. Similarly, there was no effect on amino acid digestibility when birds were fed a blend containing carvacrol, cinnamaldehyde, and capsaicin [100]. The different effects obtained in the above-mentioned trials indicate that the effect is variable and that potential improvements in digestibility are probably dependent on the ingredient composition and dosage of the phytogenic used.

Effective Dosage

It can be assumed that the effect of phytonics on gut microflora, nutrient digestibility, intestinal morphology, and, finally, performance parameters largely depends on their inclusion level in the finished feed or drinking water [112, 113]. Because of the accumulation of active ingredients, particularly in essential oils, the use of such extracts allows for the implementation of dosages below 1 g/kg of feed. In contrast, entire plants (e.g., ground herbs) or parts thereof are usually administered in higher dosages. Güler et al. [114], for example, used coriander at a dosage of 5 to 40 g/kg and reported increased feed intake and BW gain in Japanese quail. In experiments by Cross et al. [113], feed inclusion levels of different herbs, including marjoram, oregano, yarrow, rosemary, and thyme, ranged from 1 to 10 g/kg, depending on whether the entire, ground plants or their distilled extracts were used.

Depending on the physical form of phytogenic additives and on their technical possibilities on the farm, they may be applied either in the feed or in the drinking water. Supplementation of mash diets with powdered or granulated phytogenic feed additives allows for accurate inclusion levels and usually guarantees a steady supply of the active ingredients in the feed. Because of the volatility of essential oils, attention

must be paid to the thermal stability when feed is subjected to high temperatures during pelleting, extrusion, or expansion.

Application of liquid phytogenic formulas in the drinking water has the advantage of great flexibility in terms of application time and dosage. Provided that suitable dosing equipment is available on the farm, liquid phytogenic additives may be applied either continually or specifically at times of enhanced stress, such as feed change, housing, or vaccination.

IN VITRO SCREENING—ASSAY BIAS AND ASSAY DEPENDENCY

As mentioned previously, the range of AGP replacement products is quite diverse, and the search for efficacious products has relied heavily on in vitro screening assays or studies conducted with humans, rats, and other species. In vitro assays may be criticized by product end users as not accurately reflecting in vivo responses in the bird. This sentiment may be true in some cases, but in others it may partially be a reflection of assay bias attributable to media selection, duration, or substrate concentration. Nevertheless, in vitro assay techniques have become of paramount importance for biotechnological and pharmaceutical research because they allow for determination of potential mode(s) of action, allow for higher throughput for screening of product candidates, and are not influenced by environmental factors that may mask in vivo results (temperature, disease pressure) and pharmacokinetics.

Linking of in vitro techniques to in vivo viability and efficacy has been both well used and abused in the literature. One case in point is the in vitro literature regarding yeast cell wall components. Mannan-oligosaccharides (MOS) have been proposed to have a “weakening” effect on gram-negative pathogenic bacteria such as *E. coli* and *Salmonella* spp. Gram-negative pathogens (*E. coli*, *Salmonella* spp.) are able to attach to mannose residues instead of attaching to intestinal epithelial cells because of their mannose-specific lectin-like type I fimbriae [115, 116]. It has been proposed that MOS are able to bind and move pathogens through the gut without colonization, thus lessening or preventing enteric disease(s). In in vivo studies, research-

ers have not measured (and, given the current methodology, cannot measure) direct binding with pathogens, but rather have indicated reduced numbers of selected bacteria in intestinal digesta and feces [117, 118]. Worldwide, some animal feed industry personnel are claiming these health benefits, using in vitro methods to support this statement.

One method of studying the interaction of MOS with bacteria has been via the agglutination method. This method mixes bacteria with a cell wall solution, followed by visual evaluation of binding [115, 117, 119]. Critical issues with this procedure and its interpretation are the solubility of the MOS as well as the qualitative nature of the assay itself. Solubility of the MOS solution is not usually stated despite its high molecular weight, and usually results in the heavy cell wall fractions sedimenting quickly in solution. Visual appraisal of binding is highly subjective because it is not possible to evaluate whether the bacteria is actually bound, its binding affinity, or whether it is merely in proximity to the bacteria. Therefore, the use of this assay as an adequate in vitro tool is lacking because of its inability to be reproducible and quantitative across a range of products.

Another method used to study the interaction of yeast cell walls with bacteria has been via the sedimentation method. Newman et al. [120] used this procedure, assuming that bacteria that were attached to the yeast cell wall would sediment; thus, the faster the sedimentation, the better the binding between the bacteria and yeast cell wall. However, this procedure could also be criticized because all bacteria and yeasts (or yeast cell wall fractions) sediment in aqueous solutions because of their polarity and molecular weight.

A third method used to study the interaction of yeast cell walls with bacteria has been via a microtiter plate method. In this procedure, one presumes that the insolubility of the cell wall material, because of its high molecular weight, can be taken as an advantage, and the cell wall material is used to coat the wells. The wells are allowed to incubate with the test bacteria and nonbound bacteria are rinsed away, and the growth rate of bound bacteria is subsequently quantified. The accuracy and reproducibility of

binding are given by automated evaluation by optical density [121, 122].

Because of substantial variation between assays, their application should be scrutinized for product evaluation. Peer reviewers should be cognizant and diligent to make certain that all in vitro screening tests are validated for appropriateness and reproducibility, and that the relevance of results be carefully interpreted.

EFFICACY OF PROBIOTICS AND PHYTOGENICS—WHAT WE DO NOT KNOW

GIT Unknowns

Microbial Ecology and Interactions with the Host. The intestinal microbiota, intestinal tissue integrity, and gut-associated immune system are the first lines of defense against pathogens. These systems work in concert to prevent or minimize pathogen colonization and the invasion or destruction of intestinal tissues [123, 124]. The dogma is that healthy individuals develop a balanced microbial community structure that inhibits pathogen colonization without overtly stimulating the immune system. Immunological responses can be costly in terms of nutrient allocation but are necessary to rid the body of a pathogen(s) and thus keep the animal alive.

Recent advances in molecular tools have allowed us to find out more about how the microbial community in the intestine [125]. However, beyond a change in flora in different intestinal tract regions, information is sorely lacking on the horizontal (vs. vertical) distribution of bacterial flora, microbial cross-talk, and triggering or suppression mechanisms of pathogenicity for microbial pathogens.

At least some of the intestinal microbiota communicate with the intestinal epithelium, which in turn provides nutrients, binding sites, or both for these microorganisms, and fermentation products can also influence cell proliferation, differentiation, and apoptosis [124]; mucin composition [8]; and angiogenesis [126].

Therefore, when it comes to further work with feed additives targeted at eliciting effects in the intestine, consideration is needed for how the effects on the microbial environment (verti-

cally and horizontally) will affect the microbial community structure. Further, the impact on the response by the intestine, and, more important, the quantification of endogenous losses of these responses, must be elucidated.

Immunostimulation vs. Immunosuppression. Immunomodulation can be defined [127] as a change (stimulating or suppressing) in the indicators of cellular, humoral, and nonspecific defense mechanisms. Typically, the immune system is held in a homeostatic balance between immunostimulation and immunosuppression. As mentioned previously, compounds have been identified from the human literature with phyto-genic plants that have either immunostimulatory activity [e.g., ginseng, with its steroidal saponins affecting cytokine production (IL-1, IL-6, IL-12, IL-6, tumor necrosis factor- α , and interferon- γ), macrophage activation, and lymphocyte activity] [128] or antiinflammatory activity (ginko biloba, with its bioactive flavonoids and terpenes mediating production of preinflammatory cytokines) [129]. The immunostimulatory effect makes intuitive sense in that some plants have developed to produce certain compounds as preservation mechanisms to avoid being eaten after causing responses such as diarrhea or an acute phase response with fever, food intake reduction, and inflammation. Examples include compounds such as concanavalin and phytohemagglutinins, which have long been used by researchers to quantify the innate immune responsiveness.

Cost of Immunity. When it comes to the net cost of the immunological response in studies with feed additives (particularly the innate response), the collective poultry science research community has been lacking sufficient scientific originality. Rather, it is important to reevaluate experimental approaches that can encompass not only the quantity and quality of immunological responses, but also the net effects on the whole bird [130].

Because many of the feed additive products under discussion focus on improving bird performance in less than ideal sanitary environments, they have to work largely at improving the innate immune response of the bird. One of the biggest factors affecting performance loss during an acute phase response by any bird is that of feed intake suppression. The severity, du-

ration, and recovery of feed intake suppression due to the immunological naivety of the bird toward a particular pathogen can be influenced by pathogen load, virulence, bird genotype, and feed composition [131]. In some cases, the anorexia experienced during the acute phase response is necessary for some genotypes to cope immunologically with the pathogen. For example, Nestor et al. [132] compared the response of fully fed, growth-selected (F-line) turkeys with a feed-limited, growth-selected line and a random-bred line of turkeys when challenged with a high dosage of *Pasteurella multocida*. Interestingly, the mortality in the fully fed F-line of turkeys was more than 80%, but mortality of the feed-limited F-line and the random-bred line of turkeys was only 48 and 43%, respectively.

In addition to the feed intake reduction during an acute phase response, productivity is lost because of the acute phase immune response, requiring up to 10% of nutrient use that otherwise would have gone toward growth [130]. Other researchers have estimated this nutrient cost to be 1.3 times that of maintenance [133], or a daily cost of 0.27 g of ideal protein/kg of BW [134]. Therefore, when it comes to further work with feed additives targeted at eliciting effects in the intestine, these also need to consider the net effects during these subclinical infections, nutrient partitioning, and the impact on feed intake behavior of the bird.

CONCLUSIONS AND APPLICATIONS

1. Scientists have recently focused much of their work on the functionality of the intestinal tract. This emphasis has occurred 1) because of the feed ingredient cost and a desire to maximize nutrient utilization (thus also reducing emissions into the environment); 2) because of genetic improvements, whereby the first week after hatch or weaning (a critical intestinal growth period) represents a larger proportion of the life of the bird; and 3) because removal of subtherapeutic antibiotics has increased the prevalence of pathogens (including food-borne pathogens). Therefore, probiotics and phyto-genic feed additives are garnering attention.

2. Probiotic and CE products are improving their efficacy as in vitro screening for multiple traits advances. The efficiency and constraints of the regulatory or registration approval process, however, are hindering the breadth and extent of products on the market. Technological limitations, namely, tracking each probiotic strain from feed through the intestinal tract, are limiting the full understanding of bacterial interactions (with other bacteria as well as with the bird) as well as the persistence of bacteria in the intestinal tract.
3. Despite centuries of use in humans, phyto-genic products have only recently been documented in the peer-reviewed literature as influencing poultry performance. In part because of limited research in the area, full comprehension of physiological responses is incomplete. Until active components and their bioactivity are identified in poultry, product standardization will be imprecise.

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Summary

The European Union ban of antibiotic growth promoters in 2006 and the increasing interest by the consumer in wholesome and antibiotic-free food require challenging and innovative solutions for the world food supply.

Yeast derivatives including yeast cell wall components are promising alternatives to antibiotics with respect to the promotion of health and performance in livestock, based on their capacity to bind enteropathogenic bacteria and to beneficially modulate cells of the immune system.

This cumulative thesis contains manuscript's which investigate the *in vitro*, *ex vivo* and *in vivo* benefits of yeast derivatives. In chapter 1, an attempt for a standardized and reproducible *in vitro* method (microplate method) to demonstrate the binding between yeast cell wall components and enteropathogenic bacteria was made. Furthermore, selective pathogen binding could be demonstrated which led to the assumption of a certain interaction between the mannose chains of the yeast cell wall components and the mannose specific receptors of enterobacteria (mannose specific type-I fimbriae). Moreover, literature assumes also further involved, unknown binding factors which were discussed in chapter 1 and chapter 3.

In chapter 2, the immunomodulating capacity of yeast beta-glucan was studied; lymphocyte viability could be improved by beta-glucan supplementation. Finally, an *in vivo* trial with broiler chicks indicated correlations between increased performance, beneficial effects on intestinal structure (goblet cells) and white blood cells. Controversial data in literature and mode(s) of action regarding immunomodulation were reviewed and discussed.

In summary, *in vitro* and *in vivo* results suggest that yeast derivatives are promising health promoters for animal husbandry.

Zusammenfassung

Aufgrund des Verbots 2006 in der EU, antibiotische Leistungsförderer in der Futtermittelproduktion zuzusetzen, und das steigende Interesse gesundheitsbewusster Konsumenten an Antibiotika-freien und sicheren Lebensmitteln, sind innovative und herausfordernde Lösungen für die Lebensmittelindustrie gefragt wie nie zuvor. Hefederivaten, wie beispielsweise Hefezellwänden werden gesundheitsfördernde Eigenschaften wie Adhäsion von pathogenen Bakterien, Stimulation des Immunsystems und damit einhergehende Prävention von Infektionskrankheiten und Leistungssteigerung im Nutztier nachgesagt.

Die vorliegende kumulative Dissertation enthält Manuskripte, welche die gesundheitsfördernden Eigenschaften von Hefederivaten *in vitro*, *ex vivo* und *in vivo* untersuchen. In Kapitel 1 wurde eine standardisierte und reproduzierbare *in vitro* Methode (Mikrotiterplattenmethode) entwickelt um die Adhesion zwischen Hefezellwandkomponenten und enteropathogenen Bakterien zu beweisen. Zusätzlich konnte eine selektive Pathogenbindung gezeigt werden; was zu einer Annahme führte dass eine Interaktion zwischen den Mannose-Einheiten der Hefezellwand und den mannose-spezifischen Rezeptoren (Type-I fimbriae) der Enterobakterien besteht. In der Literatur wird postuliert dass weitere, unbekannte Faktoren involviert sein könnten; diese wurden in Kapitel 1 und 3 diskutiert.

In Kapitel 2 wurden die immunmodulierenden Eigenschaften von Hefe-beta-glucan untersucht; die Lymphozytenviabilität konnte durch beta-glucan Supplementierung gesteigert werden. Schlussendlich, zeigte ein Fütterungsversuch mit Hühnern Korrelationen zwischen gesteigerter Leistung, positiven Effekten an der Darmstruktur und erhöhter Anzahl an weißen

Blutzellen. Kontroverse Daten aus der Literatur und Wirkungsweise(n) im Hinblick auf Immunmodulation wurden diskutiert.

Aufgrund der *in vitro* und *in vivo* Ergebnisse können Hefederivate als vielversprechende, gesundheitsfördernde Futtermittelzusätze angesehen werden.

List of publications:

Originalbeiträge in Fachzeitschriften (SCI-Journals):

Anja Ganner, Christian Stoiber, Dominik Wieder and Gerd Schatzmayr. Quantitative *in vitro* assay to evaluate the capability of yeast cell wall fractions from *Trichosporon mycotoxinivorans* to selectively bind gram negative pathogens.
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*equally contributing

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In preparation

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(2010): Study on the inclusion level of autolysed yeast (BIOMIN PRO-Digest) as a protein source for red tilapia diets.

World Nutirtion Forum. October 15th-16th, 2010, Salzburg, Austria and BOKU Symposium für Tierernährung, April 2011

Simone Schaumberger, Sabine Masching, Anja Ganner and Gerd M. Schatzmayr.

(2010): Effects of a synergistic feed additive formulation against gram negative bacteria associated diseases.

International Pig Veterinary Science conference, Juli2010, Vancouver, Canada

Anja Ganner, Sabine Masching, Fabio Goldflus and Gerd Schatzmayr.

(2010): Yeast autolysate: microbiological and immunological properties *in vitro* and effect on piglet performance *in vivo*.

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(2010): Cytokine modulation of yeast derivatives investigated on a lipopolysaccharid-activated monocyte-macrophage cell line.

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Ganner Anja, Stoiber Christian and Schatzmayr Gerd.

(2010): Cytokine modulation of plant materials investigated on a monocyte-macrophage cell line.

World Immune Regulation meeting, March 2010 Davos, Switzerland

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(2009): Health benefits of yeast cell wall components: *in vitro* and *in vivo* investigation.

Digestive Physiology of Piglets, May 2009, Montbriol del Camp, Spain

A. Ganner, K. Erlacher, A. Acosta Aragon, A. Klimitsch, S. Nitsch and G. Schatzmayr.

(2009): *Ex vivo* effect of yeast β -glucan on lymphocyte viability and plasma IL-18 in weaning piglets.

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(2008): *Ex vivo* effect of yeast beta-glucan on lymphocyte viability of weaning piglets.
BOKU Symposium für Tierernährung, Dez. 2008, Wien

Patente

Bezeichnung: Verfahren zur Herstellung eines Futtermittelzusatzes sowie Verwendung desselben
Anmeldenummer: A8019/2010

Curriculum vitae

Personal data

Full name: Mag.rer.nat. Anja Ganner
Address: Felix Dahnstr. 38/13
1190 Vienna
Call number: 0043/650/2335699
Email: anjaganner@gmx.de
Nationality: Italian
Date and
Place of birth: Meran, 20.07.1980

Academic further education

2007 - 2011 PhD in Food- and Biotechnology at the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria
Topic: *In vitro* evaluation of yeast derivatives

1999 - 2004 Master's degree in Food science and Nutrition at the University of Vienna, Austria

08/2003 - 02/2004 Master's thesis at Massey University, Institute of Food, Nutrition and Human Health in Palmerston North, New Zealand
Topic: Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria

09/2002 - 02/2003 Student exchange programme (Erasmus) at Leicester University, United Kingdom

1994 – 1994 Pedagogical Lyceum in Meran, Italy

Career progression and practical training

Since 01/2007 BIOMIN Research Center in Tulln, Austria
Position: Research & Development Project Leader
Duties: project & budget planning, design and evaluation of scientific experiments, supervising diploma students and technicians; scientific presentations, congresses, marketing presentations

10/2006-12/2006 AKH Wien, Department of Endocrinology and metabolism, Austria
Position: Research Associate
Duties: diabetes research, literature studies

09/2006 ETH Zürich, Department of Food Microbiology, Switzerland
Duties: microbiological laboratory work

05/2005-08/2006 SY-LAB Geräte GmbH, Microbiology Department in Purkersdorf, Austria
Position: Application specialist
Duties: technical customer consulting service, training and installation of impedance instruments internally and externally worldwide, exhibition support, support for foreign agencies, technical homepage maintenance

- 11/2004 - 05/20005 AKRAS Flavours AG in Biedermannsdorf, Austria
Position: Laboratory technician for quality control
Duties: microbiological and chemical (GC) analysis of food flavors
- 08/2004 - 10/2004 University of Campinas, Sanitation and Environment Department in Sao Paulo, Brazil
Duties: water quality analysis
- 07/2004 University of Natural Resources and Applied Life Sciences, Institute of Organic Chemistry, Christian-Doppler-Laboratory in Vienna, Austria
Duties: synthesis and purification of carbohydrates
- 07/2003 - 08/2003 Laboratory for Food Analysis in Bolzano, Italy
Duties: chemical analysis of fatty acids (HPLC)
- 07/2002 - 08/2002 Dr. Schaer GmbH (gluten free diet food) in Burgstall, Italy
Duties: literature research and presentation of scientific studies
- 09/2001 Municipal Hospital, Department of diabetes and metabolism in Meran, Italy
Duties: nutrition education for diabetics

Languages

German: mother tongue; Italian/English: business fluent; Spanish: intermediate;
Portuguese: basic knowledge

Honorary office

- 2003/20004 IAESTE (The International Association for the Exchange of Student of Technical Experience) in Vienna, Austria
Duties: cooperation for exhibitions, administrative jobs
- 08/2000 “Habitat for Humanity” in Michigan, USA
Duties: social work, caring about handicapped people

Additional work experiences

- 03/2004 - 08/2004 Freelancer for DO&CO (International Event Catering)
- 06/2001 - 08/2001 Flight attendant for Laudaair (Austrian Airline Group)
- 07/1998 - 08/1998
- 07/1999 – 08/1999 Reception assistant at the Campsite „Fornella“, Lake Garda, Italy

Interests and Hobbies

Intercultural experiences in the last 10 years: South America, New Zealand, South East Asia, USA, Middle East.

Middle and long distance running; national competitions from 1991-2002 in 800m, 1500m; half-marathon.

1994-1996: Several times area champion in mountain and cross race (South Tyrol and Trentino)

1996: national champion in mountain race

Mountaineering, rock (and ice) climbing, ski-tours, mountain biking, diving.

Vienna, 15.04.2011