# Simposium: Probiotics and Prebiotics

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## Introduction

A probiotic is a viable microbial dietary supplement that beneficially affects the host, either animal or man, through its effects in the intestinal tract. The bacterial genera most often used as probiotics are lactobacilli and bifidobacteria although other groups are also represented. The health-promoting effect of lactobacilli and bifidobacteria in the colon has been mainly associated with their capacity to stimulate the immune response and to inhibit the growth of pathogenic bacteria. Nutritional studies conducted on animals and humans by different research groups show that the oral intake of probiotics can transiently improve the balance of colonic microflora allowing health benefits.

Prebiotics are non-digestible dietary supplements that selectively stimulate the growth of beneficial gut microflora and suppress potentially deleterious bacteria. Oligofructans and inulin are considered as the standard prebiotics. They are not digested in the human or animal small intestine but are selectively fermented in the colon by bifidobacteria to short chain fatty acids and lactic acid, resulting in a decreased pH in the intestine, an environment that is unfavourable to pathogenic bacteria such as *Escherichia coli* and *Clostridium perfringens.*

The most relevant health benefits attributed to the consumption of probiotics and prebiotics are: immune stimulation, enhancement of the resistance to infectious diseases, alleviation of lactose intolerance, improvement of serum lipids in hyperlipidemia, reduction of cholesterol and blood pressure, production of B-vitamins, and an increase in calcium and magnesium absorption. As a dietary supplement the fructans help prevent constipation and regulate passage time, thereby reducing the risk of colonic cancer. The combination of probiotics and prebiotics (synbiotics) has not been well studied, but their beneficial effects might be additive or even synergistic.

The most recent advances in research, production and commercialization of probiotics and prebiotics were discussed during this symposium.

## The use of probiotics and prebiotics to manage the gastrointestinal tract ecosystem

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The gastrointestinal tract (GIT) represents a small, but complex ecosystem and the resident organisms have a profound impact on the health and nutrition of the host. Probiotics (microbial food supplements) and prebiotics (nondigestible food components that selectively stimulate proliferation of beneficial bacteria) are used alone and in combination (synbiotics) as alternatives to antibiotics for managing the populations and metabolic activity of the GIT bacteria. The goal is to promote the abundance of beneficial organisms and to reduce the numbers of detrimental species. The most commonly used probiotics are lactic acid producing bacteria. Most probiotics are members of the genera Lactobacillus and Bifidobacteria, but other groups are represented. The majority of prebiotics are nondigestible oligosaccharides, with the beta-fructans receiving the greatest attention. Host responses to and the efficacy of the probiotics and prebiotics that are presently available are variable.

There is an immediate need to identify probiotics and prebiotics that provide the greatest health benefits, establish dose-response relations, determine the mechanisms of action, and understand why the responses vary so widely among individuals. Applying the tools of biotechnology will eventually lead to the development of genetically modified probiotic bacteria that provide the greatest health benefits, and the production of prebiotics that selectively promote growth of beneficial bacteria throughout the GIT. These efforts will benefit from a better understanding of the interactions among the resident bacteria, the gastrointestinal tract, and other dietary inputs, and the influences of environmental conditions and the species, age, and health of the host.

## Prebiotics: production, properties and applications

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Prebiotics are food ingredients than beneficially affect the colonic microbiota of the host by stimulating the growth or activity of certain healthy bacterial genera in the colon (mainly Bifidobacteria and Lactobacilli). Hence they might have beneficial effects on the host's physiology. In the presentation an overview was given of

prebiotics that are commercially available (fructans, galacto-oligosaccharides etc.). The various production methods, and the properties in relation with applications in food were discussed. These properties include among others solubility, stability and gelling behaviour.

The physiological effects originate from the fermentation in the colon and can be exerted at a local colonic (e.g. increased mineral absorption from the colon) and at a systemic level (e.g. lowering of serum lipid levels). An overview of these effects was given as well as the scientific evidence for these effects based on experimental animal studies and on human clinical trials with various prebiotics.

We showed how the combined properties of these food ingredients (technological and nutritional) can be used to formulate new food products with beneficial effects for the consumer. We also encompassed some legal aspects related to the use of health claims on such (functional) food products. Finally, future developments to enhance the physiological and applicative features of these food ingredients or for alternative production methods (including the use of GM-crops) were discussed.

## *Fructan metabolism in transgenic plants*

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We previously identified cDNA clones encoding fructan biosynthetic enzymes in onion. These clones were expressed in transgenic plants and shown to be fully functional. To study the enzymatic properties of fructosyltransferases we developed a rapid heterologous expression system based on the tobacco BY2 suspension cultures. These cells can be transformed with the use of *Agrobacterium* and in both callus and suspension cultures of transformed cells enzymatic activity is high. 6G-FFT from onion expressed in BY2 cells can make the whole range of fructans, when 1-kestose is provided as a substrate. The Dionex profile of the fructans made by 6G-FFT resembles that of onion, showing that 6G-FFT is able to synthesize inulin neo-series of different DP's and with different linkages. This strongly indicates that 1-SST and 6G-FFT are the enzymes responsible for the pattern of fructans found in onion.

In a collaboration with the plant breeding company Advanta and the inulin producing Sensus company the onion 1-SST and 6G-FFT genes have been transformed to sugar beet. The beets containing 1-SST accumulate 1-kestose to high levels. The beets containing both 1-SST and 6G-FFT produce the onion-type inulin neo-series, showing that 1-SST and 6G-FFT are together sufficient to produce the fructans found in onion.

## *Fructan production by microbial enzymes*

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Fructooligosaccharides (FOS) and inulin are respectively low and medium molecular weight fructans with proven bifidogenic effects when administered to humans and animals. Levan, a larger sized fructan, is less promising as a prebiotic but has other relevant food and non-food applications. Fructan production is impossible to practically achieve using chemical approaches. Commercial inulin is recovered from plants, such as chicory. Current systems for the industrial production of FOS are based either on the partial enzymatic hydrolysis of inulin, or on the transfructosylation of sucrose in bioreactors using fungal enzymes. Levan is synthesized from sucrose by bacterial levansucrases, but at present there is no marketable system for producing this polymer.

This presentation reviewed the catalytic performance of fructosyltransferases from bacteria, yeast and fungi, as well as their potential for the industrial production of different types of fructans from sucrose. A special emphasis was made in the enzyme which our group has worked with for a decade: the levansucrase (LsdA) from the plant-interactive bacterium *Gluconacetobacter diazotrophicus*. LsdA efficiently converts sucrose to FOS, mainly 1-kestose, or levan depending on the reaction conditions.

## *Analytical tools used for the structural identity of inulins in agave plants*

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Agave plants utilize the crassulacean acid metabolism (CAM) for $CO_2$ fixation. This photosynthetic mode

constitutes one of the most important physiological factors for their adaptation in arid and semi-arid environments. Fructans are the principal photosynthetic products generated by agave plants. These carbohydrates are fructose based polymers with a single glucose moiety, which can be of linear and non-linear nature. Agave plants are an economically important crop to Mexico not only because they are used on the elaboration of popular ethnic alcoholic beverages such Tequila, Mezcal, and Sotol but also, because they are a potential source of prebiotics. Due to the large amounts of fructans in agaves we studied the molecular structure of agave fructans by nuclear magnetic resonance (NMR) as well as matrix assisted laser desorption ionization (MALDI-TOF-MS) and gas chromatography-mass spectrometry (GC-MS) to establish their DP and linkage type. Fructans were extracted from six years old agave plants (*Agave tequilana*, *A. angustiforlia*, and *A. potatorum*). *Agave tequilana* presented a DP of 21 by NMR and 3 to 30 units by MALDI analyses. On the other hand, *A. angustifolia* and *A. potatorum* displayed a DP of 3 to 23 units, however fructans from *A. potatorum* showed isoform structures. The $^{13}$C-NMR and linkage types analyses demonstrated that all agave fructans are of non-linear nature. The agave fructans structures diversity assures the wide range of uses of these carbohydrates, including their potential as prebiotics.

## Development of probiotics in poultry farming

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The extensive use of antibiotics in animal farms with the purpose of promoting growth rate, increasing feed conversion efficiency and for the prevention of intestinal infections have led to an imbalance of the beneficial intestinal flora and the appearance of resistant bacteria. The use of probiotics in order to competitively exclude the colonization of intestinal pathogens has been proposed for poultry, specially after the European Commission banned certain antibiotics frequently included in feedstuffs as growth promoters.

The failure of the expected benefits of some probiotics can be attributed to the inability of the strains to colonize or survive in the gastrointestinal tract or their inability to antagonise or competitively exclude the pathogenic bacteria. In a previous study *Lactobacillus salivarius* CTC2197, a rifampicin-resistant strain isolated from the crop of chicken was selected as a potential probiotic strain on account of its high adhesiveness to chicken intestinal epithelial cells, antagonistic activity against some pathogenic bacteria and competitiveness in vivo.

The purpose of this study was to evaluate the use of *L. salivarius* CTC2197 as a probiotic in poultry, studying its ability to prevent *Salmonella enteritidis* C-114 colonization in chickens. When the probiotic strain was dosed by oral gavage together with *S. enteritidis* C-114 directly into the proventriculus in one-day-old Leghorn chickens, the pathogen was completely removed from the birds after 21 days. The inclusion of the strain in a commercial feed mixture seemed to be a good way to supply it on the farm, survival had been improved after several reinoculations in chicken feed mixture.

## Technological development and production of the probiotic PROBICID: results of the application in monogastric animals

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The composition and metabolism of the gastrointestinal microflora affect the performance of farm animals, especially young ones, subjected to environmental stress and infections in many ways. Due to the current intensive management in farm production, the animals plow very susceptible to enteric bacterial imbalance, leading to inefficient digestion, absorption of nutrients and retarded growth. To overcome these difficulties diets have usually been supplemented with antibiotics, which have indeed proved to be very effective in decreasing diarrhea and promoting animal growth. However, the appearance of resistant strains of harmful bacteria may decrease the efficacy of antibiotics. Also possible residues of antibiotics in the animal products and cross-resistance with human pathogens might also result in fearful health risks. For the above reasons there is wide interest in replacing antibiotics in diets by natural feed additives, such as live "probiotics." Diets supplemented with probiotics have been proven to prevent and cure intestinal infections and prevent death in pigs and poultry.

In this presentation we discussed different possibilities of the use of lactic acid bacteria as probiotics to improve the health and behavior of different animal species. We showed our results in the development of the probiotic PROBICID. The application of PROBICID in the feeding of poultry and pigs increased the eggs production by egg-laying hens and decreased mortality in weanling pigs.

## *Cuban experience in the production and evaluation of an enzymatic* Saccharomyces cerevisiae *hydrolysate from alcohol distillery and a competitive exclusion product as probiotics in poultry*

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During the past 10 years two Research Centers in Cuba: The University of Matanzas and the Animal Science Institute have been working in the production and evaluation of a yeast enzymatic hydrolysed from alcohol factory residual (YEH) and a competitive exclusion product (CEP) as probiotics in poultry production. YEH was produced using an enzymatic crude from a *Bacillus licheniformes* strain. It has high levels of glucan and mannan oligossacarides due to *S. cerevisiae* wall hydrolysis. The YEH was evaluated in broiler chickens and pullet hens. The mean results were obtained in physiological, immunological, productive and reproductive indicators. CEP was obtained from the ceca of healthy chicken broilers. This product was given to one day-old chickens in the first water they drunk. The chickens receiving CEP gave good results in microbiological indicators at the intestinal level, such as: an increase in *Lactobacillus*, *Enterococcus* and *Bacillus* count and a reduction of pathogenic bacteria such as coli forms and *Staphylococcus*. Also, best results were obtained in the productive indicator in the chickens treated with CEP.

## *Expression of enzymatically active* Gluconacetobacter diazotrophicus *levansucrase in* Pichia pastoris *under the glyceraldehyde-3-phosphate dehydrogenase promoter*

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Recent studies have established that the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter is an alternative to the widely used methanol-inducible *AOXI* promoter for protein expression in the yeast *P. pastoris*. In this work, the mature part of the *G. diazotrophicus* levansucrase (LsdA) fused at the C-terminus to the myc epitope and the His tag was expressed in *P. pastoris* under the control of the *GAP* promoter. The N-terminal fusion to the signal sequence of the *Saccharomyces cerevisiae* alpha factor allowed the secretion of the enzyme. A recombinant *P. pastoris* strain harbouring one copy of the *lsdA* expression cassette integrated in the genome was grown in a bioreactor using glycerol as the carbon source. After 60 hours growth, biomass reached 60 g $L^{-1}$ (dry weight) yielding 300 mg of LsdA $L^{-1}$ (4000 U $L^{-1}$) in the culture supernatant. The recombinant LsdA, most probably glycosylated, displayed catalytic properties similar to those of the natural enzyme, including the production of fructooligosaccharides and levan from sucrose or raffinose. The growth of the recombinant yeast in the presence of 5% sucrose for 60 hours increased biomass from 60 g $L^{-1}$ to 90 g $L^{-1}$. Quantitative analysis of sugars in the bioreactor culture supernatant showed that 71% of the glucose released from sucrose by the action of LsdA was consumed during the yeast growth. Likewise, about 56% (w/v) of total sugars formed were fructans, corresponding 24% to 1-kestose. The constitutive production of LsdA in *P. pastoris* did not cause toxicity to the host cells. This system potentially allows the implementation of uninterrupted batch cultures and continuous harvestings of LsdA from a single initial fermentation.

## *High-level expression of* Gluconacetobacter diazotrophicus *levansucrase in* Pichia pastoris *using the AOX1 promoter and two different signal sequences*

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We have cloned and expressed the *Gluconacetobacter diazotrophicus* levansucrase gene (*lsdA*) in *Pichia pastoris* using the methanol-controlled alcohol oxidase (*AOX1*) promoter. Two integrative vectors were constructed with two different secretion signal sequences in order to obtain efficient secretion of the protein. One vector contains the structural gene encoding the mature levansucrase gene fused to the *SUC2* gene signal sequence from *Sacharomyces cerevisiae*. In the other vector, *lsdA* is expressed with its own signal sequence. We selected the clones expressing the sucrose utilizing enzyme using a rapid screening recently established (Hernandez et al., 2002). The expresser clones corresponded to those colonies that changed the YP medium

color to yellow in the presence of the pH indicator Bromothymol Blue and supplemented with sucrose 5% and glycerol 1%. A recombinant *P. pastoris* strain containing one copy of the *lsdA* expression cassette (with *SUC2* signal sequence) integrated in the genome was fermented until high density using glycerol and methanol. Higher expression levels of the levansucrase gene were obtained (more than 1 g/L of culture) compared to a previous genetic construction (Trujillo et al., 2001). The highest LsdA activity was detected in the periplasmic fraction.

Secretion was more efficient using the *SUC2* signal sequence respect to *lsdA* signal sequence and also respect to *PHO1* signal sequence.

## *Effect of pH and aeration on levansucrase production by* Gluconacetobacter diazotrophicus *in batch fermentation*

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*Gluconacetobacter diazotrophicus* is an aerobic, nitrogen-fixing bacterium, which predominantly colonizes sugarcane and other tropical sucrose-rich crops. The bacterium secretes a constitutively expressed levansucrase (LsdA) to utilize plant sucrose. LsdA is a potential commercially important enzyme due to its high production of the bifidogenic trisaccharide 1-kestose and the polyfructan levan with different attractive applications. In an attempt to optimize the extracellular production of LsdA by *G. diazotrophicus* SRT4, the effects of critical parameters such as medium pH and air supply were evaluated in a 5-L batch reactor. The initial medium pH was examined within the range 5.0-8.0 and the dissolved oxygen (DO) was varied from 1 to 2 vvm at different agitation rates. The most favorable initial medium pH for bacterial growth and LsdA production was 6.0. The permanent aeration condition 2 vvm resulted in the highest value (64 U/ml) of LsdA secretion to the culture medium.

## *Immobilization of the* Gluconacetobacter diazotrophicus *levansucrase (LsdA) on different gels*

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Fructans are functional foods with desirable characteristics such as low calories, no cariogenicity, safety for diabetics and bifidus-stimulation. The *Gluconacetobacter diazotrophicus* levansucrase (LsdA) is a fructan-producing enzyme with potential industrial applications. From the practical point of view an immobilized enzyme is essentially superior to the free enzyme. In the present work, LsdA purified from culture supernatants of *G. diazotrophicus* SRT4 was immobilized on three different gels: Ca-Alginate, chitin and NHS-Hitrap. Each bioreactor containing 1200 U of immobilized LsdA and operating at residence time of 25 minutes was assayed for enzyme activity and stability under different reaction conditions. The effects of temperature (22, 30 and 42ºC) and sucrose concentration in the feeding fluid (200-600 g/L) on the activity and the products profile of the immobilized enzyme were determined by measuring the glucose released from sucrose and by TLC. Glucose release was maximal at 42ºC in all the bioreactors. The activity of the enzyme immobilized on Ca-Alginate and NHS-Hitrap decreased as the concentration of the sucrose solution was increased. In the case of chitin, the enzyme was most active at the sucrose concentration of 400 g/L. This result can be explained by the highest particle size of chitin that allows a faster interstitial diffusion of the substrate into the support.

LsdA immobilized on Ca-Alginate produced the highest enzymatic activity in the reactor and showed the longest biocatalyst half-life. The sucrose flow rate did not alter the profile of the enzyme products in the studied range 45-100 ml/h.

## *Prevention of bacterial vaginosis by* Lactobacillus spp. *in an experimental murine model*

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Bacterial vaginosis (BV) is a common disease in reproductive age women and is associated to important gynecologic and obstetric complications. In this work we developed an experimental murine model to study

their infection and the prevention with *Lactobacillus spp*. Inbread female adult BALB/c mice were sincronized in their hormonal cycle by estrogens administration. The basal flora in these animals was evaluated. *Lactobacillus* strain with good adherence isolated from human vagina was employed in this study. *Gardnerella vaginalis*, microorganism associated to BV, grown in human blood bilayer-Tween 80 (HBT) agar and resuspended in agarized- peptone water at 45ºC. *G. vaginalis* was inoculated intravaginally in four animals. Another group was inoculated with *G.vaginalis* at day 1 and then with *Lactobacillus spp*. strain at day 4. Another group was inoculated only with *Lactobacillus* at day 1. Vaginal washes samples were taken at 2, 5, 7 and 11 days post inoculation. They were processed for Gram stain to be analized by Nugent criteria. Animals were sacrificed in the days cited before and the number of microorganisms determined by selective plating of vagina homogenates, which were also processed for histological studies. *Lactobacillus* strain evaluated in this work was isolated until day 11. This research showed the development of an experimental BV that was protected but not treated with intravaginally inoculated lactobacilli.

## *Study of the probiotic activity of an enzymatic hydrolysate of yeast cream (*Saccharomyces cerevisiae*) in light replacement birds. Fermentative and microbiological indicators in ileum and cecum*

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A trial was carried out with 200 hybrid young female White Leghorn chicks in order to study the probiotic effect of an enzymatic hydrolysate of a distillery cream (*Saccharomyces cerevisiae*). Four treatments in a completely randomized design were used: dose I, control (0 ml of hydrolysate / kg of concentrate); dose II (25 ml/kg); dose III (50 ml/kg); dose IV (75 ml/kg). Sampling were carried out in the 14th and 18th weeks. *Lactobacillus* count in cecum was lower in the control pullet hens in the two sampling in the groups with hydrolysate and coliforms count was higher ($p<0.001$). Lactic acid in ileum was 27.7, 27.9 119,9 and 92.9 mmol/ml (14th week) and 32, 40, 49 and 75 in cecum (18th week) ($p<0.001$). The fatty acid of short chain was higher in the dosage of 50 and 75 ml in ileum (14th week) ($p<0.001$). The pH was lower in ileum and cecum in the dosage of 50 and 75 ml ($p<0.001$). It was concluded that this product has a probiotic effect due to the improvement in the indicators studied. It is recommended to carry out further studies in the use of this hydrolysate in the category of young female White Leghorn chicks.