



Evaluation of chitoligosaccharides effect upon probiotic bacteria

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ABSTRACT

The main objective of the present study was to evaluate the antibacterial effect – through the determination of minimum inhibitory (and lethal) concentrations, as well as the possible prebiotic potential of chitoligosaccharides (COS) – through the determination of growth curves, on *Bifidobacterium animalis* Bb12, *Bifidobacterium animalis* Bo and *Lactobacillus acidophilus* Ki. Atomic force microscopy was further used to obtain high resolution images of COS effects upon the cell morphology. Our results demonstrate that COS do not stimulate the growth of those strains, neither the strains are capable of using COS as a primary source of carbon. Analysis of morphology when exposed to inhibitory/bactericidal concentrations, suggested that COS do not exert any direct damage upon the bacteria structure, instead the bacteria are apparently covered by COS, which likely prevent nutrient uptake.

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1. Introduction

Over the past decade, many food products have been launched with prebiotic compounds and probiotic microorganisms added – which are known for their functional properties [1]. The term “prebiotic” was introduced by Gibson and Roberfroid (1995) – who defined it as “a non-digestible food ingredient, which beneficially affects the host by selectively stimulating the growth and/or activity of one (or a limited number) of bacteria that already exist in the colon” [2]. Furthermore, prebiotics can inhibit growth of some pathogens, thus assuring additional benefits to the host's health. Probiotics on the other hand, are live microbial ingredients, which beneficially affect the host health via colonization of the lower intestine – and which usually belong to the *Bifidobacterium* or *Lactobacillus* genera [3].

Inulin and such oligosaccharides, as fructooligosaccharides (FOS), are known examples of prebiotics [4,5]. More recently, oligosaccharides obtained from chitosan – i.e. chitoligosaccharides (COS), as well as low molecular weight chitosan, have been claimed to also exhibit prebiotic effects [6]. Additional studies also revealed important biological properties, such as lowering of blood cholesterol levels [7] and blood pressure [8], antimicrobial,

antitumor, antioxidant and radical scavenging activities, and immunostimulatory effects [9–11].

COS are partially hydrolyzed products of chitosan, a biopolymer composed of β -(1-4)-linked *N*-acetyl-D-glucosamine and deacetylated glucosamine units [12]. Interest has been recently focused on their solubility in acid-free aqueous media, as opposed to high molecular weight chitosan. Furthermore, such oligomers have been shown to have more potential than chitosan as a nutraceutical additive, since COS are easily absorbed through the intestine, quickly getting into the blood flow and having systemic biological effects in the organism [13,14]. As implicit in definition of prebiotics, they should increase growth of probiotic bacteria – yet, existing experimental data have failed to be definitely conclusive.

Atomic force microscopy (AFM) is a highly versatile microscopy technique that is particularly well suited to the study of microorganisms, because it combines a greatly improved resolution (when compared to optical microscopy) with little or no sample preparation required [15]. Furthermore, when compared with conventional scanning electron microscopy, samples can be studied in a more natural state, as there is no requirement to scan in vacuum, or for any type of conductive coating. Therefore, AFM has been widely applied to studies of bacterial morphology [16–19]. Despite not being suited to inspection of large samples, and unable of high throughput rates due to slow acquisition time, AFM can generate images ranging from tens of micrometers to tens of nanometers in size and hence, has enough resolution to image many bacteria at once, as well as to observe small changes in cell morphology, or even nanometer-scale features such as

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spore rodlets (3 nm in diameter) [20] or bacterial division septa [21,22].

In view of the above, this study focused on evaluation of the effect of COS upon the growth of three probiotic bacteria (i.e. *Bifidobacterium animalis* Bb12, *Bifidobacterium animalis* Bo and *Lactobacillus acidophilus* Ki) [23–25]. AFM imaging was used to obtain high resolution images of the effect of the COS on the bacterial morphology.

2. Materials and methods

2.1. Chitooligosaccharides and microorganisms

Two COS mixtures were purchased from Nicechem (Shanghai, China) with molecular weight 1.763 ± 0.7 and 4.134 ± 0.6 kDa (named COS3 and COS5, respectively) and similar degree of deacetylation (65%). For the preparation of the solutions, as-received COS were dissolved in water to 2.5% (w/v) and the pH was adjusted to 5.8 with 10 M NaOH. After stirring overnight, the solutions were autoclaved at 120 °C for 15 min.

The strains used were *B. animalis* Bo, *L. acidophilus* Ki and *B. animalis* Bb12. These microorganisms were grown at 37 °C in de Man-Rogosa-Sharpe (MRS; Biokar, France) broth for 48 h. MRS was supplemented with filter-sterilized 0.5 g/L of L-cysteine-HCl (Fluka, Switzerland), and incubated under anaerobic conditions.

2.2. Determination of minimum inhibitory and lethal concentrations

In order to establish a boundary between antimicrobial and prebiotic concentrations, the MIC and MLC were duly determined. Minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of COS at which microorganisms cannot grow in Muller–Hinton (M-H) broth (Lab M, UK), supplemented with filter-sterilized 0.5 g/L of L-cysteine-HCl. Experimental concentrations of 1.0, 0.50, 0.25, 0.15, 0.125, 0.10 and 0.05% (w/v) were used for that determination. A fixed volume of solution, at the appropriate concentration, was mixed with the broth in a test tube, which was then inoculated with 10^5 cfu/mL of each test bacterium; microbial growth was monitored via turbidity, by 48 h of incubation at 37 °C under anaerobic conditions. On the other hand, MLC was determined as the lowest concentrations of COS at which microbial growth was prevented, and the initial viability was in addition reduced by at least 99.9% within 48 h; it was determined by inoculation of 100 µL-aliquots of negative tubes (i.e. with absence of turbidity in MIC determination) on MRS agar, supplemented and incubated as described above, using the spread-plate technique.

2.3. Assessment of the prebiotic activity of COS

The prebiotic effect of COS3 was evaluated through the *B. animalis* Bo, *L. acidophilus* Ki and *B. animalis* Bb12 growth. Assays were carried out in Man-Rogosa-Sharpe (MRS) broth culture media (supplemented with 0.5 g/L of L-cysteine-HCl), containing three different concentrations of COS – 10, 100 and 1000 mg/L. Other culture media, with the same composition than the MRS, but where glucose as a carbon source was replaced by COS (at the same above mentioned concentrations), was used to evaluate the *B. animalis* Bo, *L. acidophilus* Ki and *B. animalis* Bb12 growth. COS was filter-sterilized and added to the sterilized media to give the mentioned final concentrations. The media were named: M1 – MRS broth; M2 – MRS without glucose; M3 – MRS broth plus 1.0% (w/v) of COS3; M4 – MRS broth plus 0.10% (w/v) of COS3; M5 – MRS broth plus 0.010% (w/v) of COS3; M6 – MRS broth without glucose, plus 0.10% (w/v) of COS3; and M7 – MRS broth without glucose, plus 0.010% (w/v) of COS3. The bacteria were inoculated (ca. 10^6 cfu/mL) in the

Table 1

Minimum inhibitory (MIC) and lethal (MLC) concentrations (%) of COS3 and COS5 upon *L. acidophilus* Ki, *B. animalis* Bo and *B. animalis* Bb12.

Strain	Test	COS3 (%)	COS5 (%)
<i>Bifidobacterium animalis</i> Bo	MIC	0.25	0.15
	MLC	0.50	0.50
<i>Lactobacillus acidophilus</i> Ki	MIC	0.50	0.50
	MLC	0.50	0.50
<i>Bifidobacterium animalis</i> Bb12	MIC	0.50	0.25
	MLC	0.50	0.50

different culture media and incubated with shaking anaerobically for 48 h at 37 °C. Samples were taken at different time periods, 1, 3, 5, 7, 8, 13, 24, 36 and 48 h, plated by the spread technique in MRS agar (supplemented with 0.5 g/L of L-cysteine-HCl) and incubated anaerobically at 37 °C for 48 h. Microbial growth was determined by plate counting, and expressed as log cfu/mL. The pH changes of the broths were also determined at the same sampling periods, using pH/ISE meter (Orion Research, Inc., America). All measurements were performed in triplicate parallels and repeated at least twice.

2.4. Preparation samples and AFM analysis

The effect of COS on the bacterial cell surfaces was examined by AFM. Samples were prepared by applying 40 µL of bacterial suspension without treatment (control) or treated with COS (1.0, 0.1 and 0.01%) onto a clean glass surface, followed by air-drying. The samples were incubated likewise to the antibacterial assays. The samples were then gently rinsed with deionized water to remove salt crystals, and air dried again before analysis. AFM analysis of the control samples showed that *B. animalis* Bo did not react well to such preparation, and showed distorted cell morphology, rather than the expected rod shape. The other strains, however, showed the expected rod-like cell shape in all samples. It was hypothesised that *B. animalis* Bo was more sensitive to aerobic conditions used for preparation of AFM samples than the other strains, so AFM samples were prepared and dried under anaerobic conditions.

AFM imaging was carried out with a Veeco Multimode IVa atomic force microscope (Veeco, Santa Barbara, CA, USA), equipped with a j-type scanner (ca. 100 µm × 100 µm × 5 µm scan range). Bacteria morphology studies were carried out in tapping mode in air, using silicon cantilevers with a resonant frequency approximately 150 kHz (MikroMasch, Tallinn, Estonia). Two independently produced samples were analyzed, and several different areas were studied on each sample, but only characteristic images are shown here.

2.5. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) where time and concentration were considered factors. Comparisons of the means were conducted using a Tukey's test. All statistical analyses were performed using the SPSS package program version 16.0, and differences were considered significant at $P < 0.05$.

3. Results and discussion

COS have been proven to possess strong antibacterial activity upon growth of a wide variety of microorganisms, including Gram-positive and -negative bacteria, yeast and molds [26]. In order to elucidate this effect upon bifidobacteria (and lactic acid bacterium), and thus to conclude on whether COS are potential prebiotic agents, MICs and MLCs were assessed. The results are depicted in Table 1.

It can easily be observed that MIC and MLC values did not vary considerably with the bacterium or with COS MW. In general, at

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