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Effects of Different Oligosaccharides on Growth of Selected Probiotic Bacterial Strains

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Abstract

Objective: To assess if prebiotics at different concentrations can accelerate the growth of selected probiotic bacterial strains.

Materials and methods: *Enterococcus (E.) faecium* NCIMB 10415 E1707 was chosen as it is the most common probiotic strain used for small animals. In addition, *E. faecium* NCIMB 30183, *Bifidobacterium (B.) longum* NCIMB 30182 and *B. infantis* NCIMB 30181 were tested. They were grown in 96-well plates and growth was assessed by optic density at 600 nm, using a bacterial plate reader. The prebiotics used were Fructo-Oligosaccharides (FOS), mannan oligosaccharides (MOS) and Preplex® (a combination of FOS and gum Arabic available in a commercial synbiotic product for small animals). Initially, addition of inulin was also planned but not achieved due to technical difficulties. The prebiotics were used at 20 mg/ml, 10 mg/ml, 1 mg/ml and 0.1 mg/ml, respectively. Growth rates were calculated, technical and biological repeats averaged and compared between prebiotic treatments for each strain using ANOVA.

Results: Growth of *E. faecium* NCIMB 10415 E1707 was not improved by any additive. *E. faecium* NCIMB 30183 grew significantly faster with the highest concentration of Preplex®. Both *Bifidobacterium* strains showed significant acceleration of growth with Preplex® and FOS, but only *B. infantis* showed a dose-effect.

Conclusion and clinical significance: Prebiotic additives have to be chosen depending on the probiotic strain. The *E. faecium* strain most commonly used in small animals was not influenced by any of the prebiotics used, even though commercially available as a synbiotic. The growth of Bifidobacteria was accelerated with commonly used prebiotic oligosaccharides. Interestingly, the addition of gum Arabic seemed to have a stronger effect on growth acceleration than FOS alone. The information gained might have implications for the design and production of pre- and probiotic formulations for small animals in the future.

Keywords: Bifidobacterium; Enterococcus; Gastroenteritis; Growth rate; Synbiotics

Introduction

A wide variety of veterinary probiotic products is available over the counter. They are popular for the treatment of several conditions in small animals, mostly related to the gastrointestinal (GI) tract, where there is some evidence that they can alleviate symptoms of acute gastroenteritis [1,2]. There have also been several attempts to assess potential health benefits of probiotics in chronic GI conditions in small animals [3-6]. Even though a probiotic is defined as "a live organism which, if administered in adequate quantities, confers a health benefit to the host" proof of efficacy in specific conditions is often lacking, as most of these products are sold as health/ food supplements, not as drugs [7]. More comprehensive reviews on their clinical efficacy in small animals can be found elsewhere [8]. The term prebiotic is typically used to describe "non-digestible food ingredients that stimulate the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" hence they are often dietary fibres [9]. A number of studies have shown that selective fermentation of indigestible fibres induces a variety of microbiological and metabolic changes in intestinal microbiota, which might benefit the host [10]. So far, several dietary fibres and oligosaccharides have been discussed as (candidate) prebiotics [11].

The most widely studied probiotic for small animals is *Enterococcus (E.) faecium* however, other lactic acid producing bacteria, e.g. Bifidobacteria are also frequently used in probiotic preparations for humans and tested for companion animal consumption [2-5,12-14]. The aim of this study was to test the growth properties of 2 different strains of *E. faecium* and Bifidobacteria, respectively, with the addition of different prebiotics *in vitro* to potentially inform future decisions about combining pre- and probiotics.

Materials and Methods

Probiotic microorganisms

Two different *E. faecium* strains from 3 different sources were tested. Oralin® powder for animals (Chevita GmbH, Pfaffenhofen, Germany) and Synbiotic D-C® (Protexin, Probiotics Ltd., Somerset, UK) both contain *E. faecium* NCIMB 10415 E1707. In addition, another strain of *E. faecium* NCIMB 30183 as well as *Bifidobacterium longum* NCIMB 30182 and *Bifidobacterium infantis* NCIMB 30181 were acquired from the National Collection of Industrial Food and Marine Bacteria (NCIMB Ltd., Bucksburn, Aberdeen, UK) in a freeze-dried form. Starting cultures were prepared from both products containing the *E. faecium* E1707 in

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5 ml of nutrient broth no. 1 (Sigma-Aldrich, Dorset, UK) at standard incubation conditions (orbital shaker at 37°C and 225-250 rpm). The freeze-dried cultures were revived as by NCIMB's instructions: Briefly, the glass vials were carefully opened using a diamond cutter, 0.5 ml of nutrient broth were added and the cultures incubated at 37°C and 250 rpm overnight. They were sub-cultured with a larger amount of nutrient broth for another 4 h.

All strains were then plated onto nutrient agar plates (Sigma-Aldrich), incubated at 37°C overnight and stored at 4°C until further use. In addition, glycerol stocks were prepared from each strain and stored at -80°C. Fresh secondary cultures in nutrient broth were obtained from the nutrient agar plates for each growth assay (Table 1).

Prebiotic additives

The prebiotics used at the respective concentrations can be found in Table 1. All of them were provided by Probiotics Ltd. (Somerset, UK). The concentrations of prebiotic additives were chosen empirically, as there is no data available on their optimal concentration to stimulate probiotic growth *in vitro* or *in vivo*. This is why a wide range of concentration was tested. The highest concentration (20 mg/ml) could not be used for one of the prebiotics (mannan oligosaccharides [MOS]), as it would leave the culture too cloudy to perform growth measurements based on optic density (OD, see below). In addition, an attempt was made to test inulin as a prebiotic additive at all concentrations. However, it was not soluble in the nutrient broth used; hence results of these experiments were considered unreliable and are thus not reported.

Bacterial growth curves and determination of growth rates

Growth curve analysis was based on OD measured at 600 nm. Bacteria was grown in 200 µl per well of a flat-bottomed 96-well plate and OD was measured with the Spectramax® 340PC 384 plate reader (Molecular devices, Wokingham, UK) every 5 min for 16 h. Each concentration for each bacterial strain was run in at least 4 technical and 3 biological replicates. OD data of technical replicates were averaged and plotted against time. The slope of the exponential section of the bacterial growth curve was determined by linear curve fitting. Slopes of the three biological replicates across all prebiotic additives were analysed using ANOVA (Graph Pad Prism 5, La Jolla, California, USA). Statistical significance was set at $p<0.05$.

Results and Discussion

Growth characteristics of probiotic strains without any additives were similar (Figure 1). Specifically, there was no difference between the *E. faecium* NCIMB 10415 from two different sources (data not shown), hence only the one from the Synbiotic D-C® product was used in further experiments. Exponential growth commenced between 60 and 90 min. After 200-245 min, growth plateau was reached (Figure 2). The chosen prebiotic additives influenced growth of the examined bacteria in a strain-specific and dose-depending manner. For *E. faecium* NCIMB 10415, none of the additives had a significant effect on growth rates (Figure 2A). For *E. faecium* NCIMB 31083, only the highest concentration of Preplex® accelerated growth significantly compared to baseline growth ($p<0.001$ (Figure 2B). Both Bifidobacterium strains tested showed acceleration of growth with Preplex® and FOS. *B. longum* showed a dose effect for these two prebiotics, with a significant effect of the highest concentrations: growth was accelerated with 10 mg/ml ($p<0.05$) and 20 mg/ml ($p<0.001$) of Preplex® added, as well as with 10 and 20 mg/ml of FOS (both $p<0.001$) (Figure 2C). Growth of *B. infantis* was enhanced by nearly all concentrations of FOS used (0.1 mg/ml $p<0.01$; 10 mg/ml $p<0.05$; 20 mg/ml $p<0.01$), and also with the lowest concentrations of Preplex® (0.1 mg/ml and 1 mg/ml $p<0.05$) (Figure 2D).

Prebiotic	Concentration tested
Preplex (FOS+gum Arabic)	20 mg/ml
	10 mg/ml
	1 mg/ml
	100 µg/ml
FOS	20 mg/ml
	10 mg/ml
	1 mg/ml
	100 µg/ml
MOS	1 mg/ml
	100 µg/ ml

FOS: Fructo-Oligosaccharides; MOS=Mannose Oligosaccharides

Table 1: Prebiotics used in the present study.

The present study suggests a strain-specific beneficial effect of the tested prebiotics. Interestingly, Preplex® seemed to give most consistent results. As it is a combination of FOS and gum Arabic; and improved growth was seen with Preplex®, but not with FOS alone, it is tempting to speculate that the prebiotic effect is due to the gum Arabic. This component has been shown to have some prebiotic effects in human *in vivo* studies but is largely untested in small animal veterinary medicine. Experimental studies revealed that it can modulate intestinal absorption counteract the effects of secretory toxins and potentially modulate, intestinal inflammation via down regulation of NFκB, a master regulator of inflammation, and modulation of NO production in the intestinal epithelium [15-21]. Further studies into this soluble fibre in the context of probiotic growth and intestinal health in animals might be warranted.

The most widely available probiotic strain for small animals (*E. faecium* 10415) did not show significant growth acceleration with any of the prebiotics, which simply confirms that prebiotic additives have to be chosen carefully and specifically if the desired effect is to enhance growth of a specific probiotic strain. As far as the authors are aware, there is no published data available on how to enhance probiotic *E. faecium* growth neither *in vitro* nor *in vivo*. Hence comparison of the present findings with other studies is not possible. Further studies investigating the interactions of *E. faecium* with prebiotics and other substances should be encouraged, as this might help to understand its role as a probiotic in veterinary medicine. So far, clinical trials with *E. faecium* as a probiotic with our without prebiotics have not been encouraging which might partially be due to the fact that likely high numbers of this strain are needed *in vivo* to elicit an effect (personal observations of the authors), but also potentially because a way to enhance *E.faecium* growth *in vivo* and *in vitro* have not yet been identified in these scenarios [3-5]. In only one study that was assessing the effect of another probiotic formulation; an increase in faecal enterococci was observed in dogs [22].

Overall, the present results show that the administration of prebiotics in certain concentrations might encourage the growth of certain bacterial strains (especially bifidobacteria), which can either be administered simultaneously as synbiotics, or which are already present in the GI tract as part of the endogenous microflora [8]. Bifidobacteria are part of the canine GI microbiota and certain strains might be classified as probiotics [8,13,23]. Bifidobacterium animals AHC7 has been shown to protect against pathogen-induced NFκB activation *in vivo* and was able to reduce both the need to administer antimicrobials and the overall duration of illness in acute idiopathic diarrhoea in dogs [24,25]. Based on this information and the results presented here, bifidobacteria might be interesting probiotic candidates

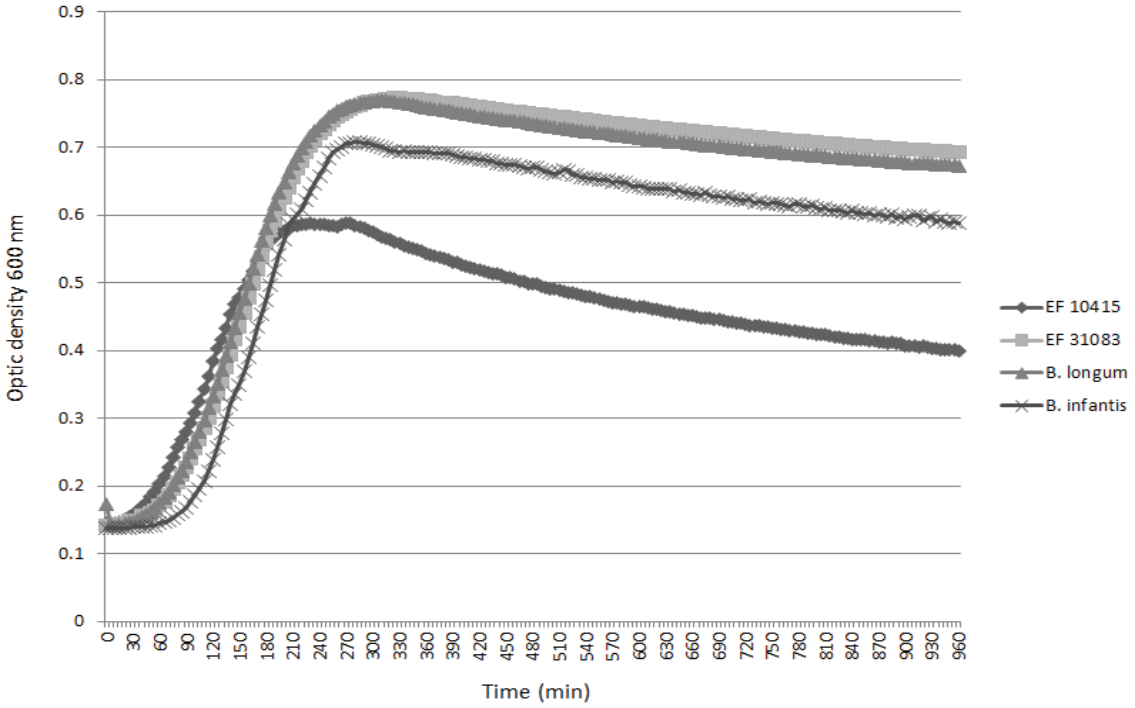
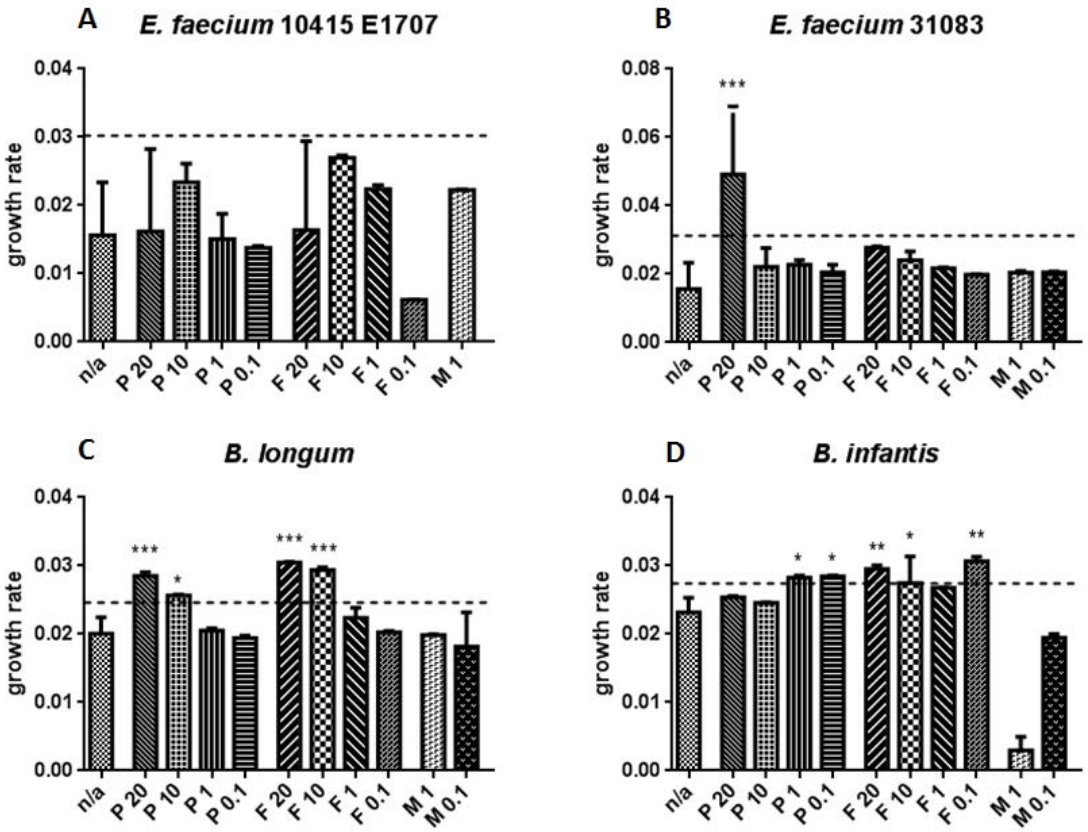


Figure 1: Growth curve for the probiotic strains used in the present study (without any additives).



n/a=no additive, P=Preplex ® (Fructo-Oligosaccharides [FOS] and gum Arabic). F=FOS, M=mannose oligosaccharides. *p<0.05, ** p<0.01, *** p<0.001 (in comparison to n/a culture). Numbers behind letters indicated used prebiotic concentrations in mg/ml

Figure 2: Growth rates for the 4 different probiotic bacterial strains with different prebiotics. The dashed line represents 2x standard deviation of the untreated cultures.

in small animals, especially as their growth seems to be easily enhanced with common prebiotics like FOS and gum Arabic.

Direct translation of the findings of the current study into an *in vivo* situation or extrapolation of prebiotic concentrations to animal feed might be challenging. Even though there is ample evidence that the addition of fibre to diets changes food fermentability increases the production of short-chain fatty acids (SCFA) and other bacterial metabolites in the colon and changes the intestinal microbiota composition in dogs the type of prebiotic or fibre studied varies greatly. Additional inconsistencies in study design, concentrations of additives and outcome measures/ techniques used make comparisons between studies difficult. Especially FOS has not been tested extensively as prebiotics in small animals, but some preliminary data are available [26-31]. In one study, addition of 1-3 g of FOS to animal feed (total dose) did not have an effect of SCFA and other metabolite production [32]. In another, short-chain FOS produced the largest decrease in faecal pH and a significant increase of acetate and propionate in faecal inoculum from dogs, which was associated with a significant increase of bifidobacteria [29]. This potentially ties in with the observations made in the present study, making the effects of FOS on bifidobacteria in the canine intestinal microbiota an interesting topic of further research.

It has to be taken into account, however, that *in vitro* bacterial growth experiments with a single strain and very high concentrations of prebiotics cannot be directly correlated with the *in vivo* situation, where a plethora of different bacterial (and other microbial) species compete for nutrients and ecological niches. Other factors (acid stability, capability to adhere to the intestinal mucus layer) might also influence the survival and expansion of externally administered probiotic strains in the gut. Thus, the results of this study have to be interpreted carefully, and only *in vivo* experiments in the target species (feeding experiments that assess changes in the microbiota composition with modern high-throughput sequencing methods) will ultimately determine if a beneficial effect can be translated into a clinical situation.

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Conflict of Interest

This work was part of a PhD project carried out at the Royal Veterinary College, London, UK, which was supported by a stipend from the BBSRC in collaboration with Probiotics International Ltd., Somerset, UK. The sponsors have seen and approved the manuscript, but had no input or influence on the design of neither the study, nor the interpretation or discussion of results.

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