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Development of a synbiotic potential score as a quantitative approach for selecting putative synergistic strain-prebiotic candidates

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Abstract

Aims **Synbiotics are increasingly being examined for their potential to enhance host health. Synergistic synbiotics are specifically designed such that the prebiotic component is selectively utilized by co-administered microorganisms, thereby providing enhanced benefits to the host. In this study, we propose a synbiotic potential score (SPS), a quantitative, high-throughput method for assessing bacterial proliferation on selected dietary fibres that incorporates growth curve values. This score was applied to both rich and minimal media to assess the utilization of difficult-to-digest fibres, such as resistant maltodextrin (RM).**

Methods and results **The SPS score was first calculated for eight putative probiotic strains grown on short chain fructooligosaccharides (scFOS), RM, and their combination. *Lactiplantibacillus plantarum* DSM 20205, *Bifidobacterium breve* JCM 7017, *Bifidobacterium longum* APC 1472, *Bifidobacterium adolescentis* DSM 20083, and *Bifidobacterium animalis* subsp. lactis ATCC 27536 scored highest for scFOS, whereas their growth on RM compared to that on media without carbohydrates was minimal. Four strains were selected for further experiments in minimal MRS medium. All four bacteria demonstrated improved utilization of RM, as indicated by a higher SPS score, suggesting that minimal media better supports the specific utilization of target prebiotics.**

Conclusions **Our approach reduces confounding factors, enabling clearer insights into prebiotic–probiotic interactions. This supports the systematic selection of effective strain-prebiotic pairs, guiding targeted synbiotic development for personalized nutrition and improved health outcomes. Future studies should validate our approach across multiple strains within the same species and using *in vivo* models.**

Impact statement

This study introduces an innovative synbiotic potential score designed to assess interactions between prebiotics and probiotics. This high-throughput technique marks a notable improvement over conventional methods, enabling the swift evaluation of numerous prebiotic-strain interactions while simplifying experimental procedures. It offers an effective platform for identifying the most promising prebiotic and probiotic combinations for further testing in mechanistic functional assays. Notably, although growth capacity serves as an initial filter, it alone does not determine synbiotic effectiveness *in vivo*.
Furthermore, the study incorporates the use of more minimal media. While minimal media based on MRS components have been utilized before, the novelty of this research lies in the systematic integration of a defined minimal media with high-throughput growth curve analysis and the synergistic prebiotic score. This integrated approach allows for a more accurate, scalable, and comparable evaluation of prebiotic utilization, directly addressing a critical gap in synbiotic formulation strategies.

Keywords Synergistic synbiotics, Microbiota, Fibres, Bifidobacterium, Synbiotic potential score (SPS)

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Introduction

In recent years, a growing understanding of the human gut microbiome has highlighted its pivotal role in shaping overall health by modifying gut composition (Rajoka et al. 2017, Sandhu et al. 2017, Berding et al. 2021, Wasiewska et al. 2024). Prebiotics, defined as substrates selectively utilized by host microorganisms that confer health benefits, have been extensively studied for their capacity to promote host health (Hutkins, 2025). The well-established health benefits of consuming prebiotics include increased mineral absorption and reduced risks of cardiovascular disease, obesity, diabetes, osteoporosis, and gut inflammation (Whisner et al. 2013, 2014, 2016, Rosa et al. 2021, Ribeiro et al. 2022).

More recently, synbiotics—defined as "A mixture, comprising live microorganisms and substrate(s) selectively utilized by host microorganisms, that confers a health benefit on the host" have emerged as complementary or synergistic enhancers of health at the host–microbe interface (Swanson et al. 2020). Synergistic synbiotics are specifically formulated to include a substrate, often a prebiotic, that is utilized by the probiotics within the formulation, whereas complementary synbiotics contain prebiotic and probiotic components that function interdependently (Lee et al. 2024).Growing evidence suggests that synergistic synbiotics, in which the prebiotic specifically enhances the growth or function of the included probiotics, may be used to support host health or as a therapeutic approach in the clinical management of various diseases by modifying the microbiome (Gurry 2017, Lee et al. 2024, Singh et al. 2024).

The selection of a compatible combination of live microorganisms and substrates, with an emphasis on efficient prebiotic digestion by the candidate probiotic, is the first step in the identification and development of effective synergistic synbiotics. However, not all prebiotic substrates are equally utilized by every bacterial strain, and the variation in bacterial processing of these substrates emphasizes the need for efficient methods of systematic evaluation.

Bifidobacteria have well-characterized preferences for certain carbohydrates, with fructo-oligosaccharides (FOS) and inulin consistently reported as the most preferred carbon sources, which are efficiently metabolized to yield acetate and lactate (O'callaghan and Sinderen Van 2016). Other readily utilized substrates include galacto-oligosaccharides (GOS), human milk oligosaccharides (HMOs, particularly in infant-associated strains), and simple monosaccharides such as glucose. In contrast, structurally complex carbohydrates, such as resistant starches or branched fibres like RM, are often poorly fermented by single strains and may require cooperative degradation by microbial consortia (e.g. Ruminococcaceae) (Kelly et al. 2021).

Existing *in vitro* methods for evaluating the efficacy of substrate-prebiotic and probiotic combinations are insufficient for evaluating strain-specific growth differences. Previous studies have established methods for quantitatively assessing the growth of various bacterial strains on different prebiotics to select potential synbiotic combinations (Fuhren et al. 2020). These methods typically compare the growth of selected probiotic strains on prebiotics to their growth on glucose using colony forming units (CFU) with enumeration at certain time points (e.g. 24/48 or 72 h) or the highest OD600 value (Bielecka et al. 2002, Grimoud et al. 2010, Fuhren et al. 2020, Śliżewska and Chlebicz-Wójcik 2020). A more comprehensive method was proposed by Huebner et al. (Huebner et al. 2007), who described the so-called prebiotic activity score (PAS) as a tool to evaluate bacterial growth on prebiotics relative to enteric bacteria, such as *E. coli*. The score was calculated based on the viable cell counts of the tested strains grown with different prebiotics at a single time point (24 h). Subsequently, other studies have adapted this method, sometimes modifying the time point before plating and counting to 48 or 72 h (Rezende et al. 2022, Alves-Santos et al. 2023).

The use of CFU at a specific time point does not consider the differences in growth characteristics between different bacterial strains that may be in different growth phases at the time of enumeration. Consequently, a low bacterial count could be caused by insufficient growth on a selected substrate, as well as being in the death phase at the time of enumeration, leading to biased conclusions. Despite this limitation, several studies have adopted this method by employing an incubation period of 24–96 h before plating and subsequent viable count determination (Fissore et al. 2015, Andreani et al. 2021). In addition, to calculate the PAS score of the putative probiotic bacteria on the potential prebiotic substrate, it was divided by the growth score of the commensal *E. coli*. This suggests that if an *E. coli* strain grows on a tested substrate, the substrate cannot be considered a prebiotic. However, this is not in line with the most recent definition of a prebiotic, which states that any substrate can be classified as a prebiotic if it supports any bacterial strain that confers health benefits to the host (Gibson Glenn et al. 2017). Certain *E. coli* strains have recently been made commercially available as probiotic products, suggesting that they can also confer health benefits (Sonnenborn 2016, Wassenaar 2016). Therefore, the use of *E. coli* as a reference strain in PAS calculations should be reconsidered.

Resistant maltodextrin (RM), also referred to as resistant starch, is a dietary fibre derived from starch sources such as corn, potatoes, and wheat (Astina and Sapwarobol 2019). Unlike regular maltodextrin, which is rapidly digested and absorbed in the small intestine, RM is not completely degraded there (Baer et al. 2014). Instead, RM passes undigested to the colon, where it is fermented by gut microbes, exerting beneficial health effects (Baer et al. 2014). Several studies have demonstrated the health benefits of RM, such as reducing the risk of obesity, insulin resistance, and metabolic syndrome, and increasing mineral absorption, digestive function, immune function, and bone health (Boler et al. 2011, Hashizume et al. 2012, Timm et al. 2013, Jakeman et al. 2016, Whisner et al. 2016, Costabile et al. 2017, Burns et al. 2018, Sorndech et al. 2019). In addition, RM showed synbiotic potential in a randomized clinical study when combined with *Lactobacillus rhamnosus* GG (Costabile et al. 2017). Specifically, RM has been found to promote the growth and associated metabolic activity of various members of the genus *Bifidobacterium* and the formerly unified *Lactobacillus*, which are known for their positive effects on gut health and anti-inflammatory properties (Burns et al. 2018, Sorndech et al. 2019). This outcome may have resulted from the direct or indirect metabolism of RM by the organisms, potentially utilizing free sugars supplied by other bacterial consortia metabolizing the RM. The human trials also showed increased production of certain short-chain fatty acids (SCFAs) in the gut, such as butyrate, which supports gut barrier function and may reduce inflammation (Fastinger et al. 2008, Sorndech et al. 2019, Arroyo et al. 2023). Emerging evidence also suggests that RM's impact on the microbiome may confer indirect metabolic benefits, including improved insulin sensitivity and reduced systemic inflammatory

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markers (Aliasgharzadeh et al. 2015, Astina and Sapwarobol 2019, 2020). These effects are thought to be mediated, in part, by the role of the gut microbiome in energy metabolism and inflammation (Nieuwdorp et al. 2014).

Fructooligosaccharides (FOS) are another group of well-studied prebiotics that occur naturally in plants, such as garlic, asparagus, bananas, and artichokes (Aliasgharzadeh et al. 2015), and are commonly added to food products, such as yoghurt or baby formula (Singh et al. 2017). FOS is composed of fructose monomers covalently bonded via β1–2 glycosidic links to form an oligomeric structure with a terminal glucose unit. Similar to RM, FOS is not digested by host enzymes and may be metabolized by gut commensals in the lower small intestine (ileum), caecum, or colon. FOS also promotes the growth of various members of the genus *Bifidobacterium* spp. and formerly unified *Lactobacillus* spp. (Gibson and Wang 1994, Kaplan and Hutkins 2000, Mahalak et al. 2023, Xu et al. 2025). FOS-containing diets are associated with several health benefits, such as increased calcium absorption into the bone, regulation of the immune system response, lipid metabolism, and glucose homeostasis (Caetano et al. 2016, Krupa-Kozak et al. 2016). The synbiotic potential of FOS with selected bifidobacteria or lactobacilli has also been extensively studied in recent years (Nazzaro et al. 2008, Anand et al. 2018, Skrzydło-Radomańska et al. 2020). However, more evidence is needed to establish the health benefits of these combinations, particularly regarding strain specificity and their effects under different growth conditions.

In the current study, eight strains representing selected bifidobacteria and lactobacilli were screened for their ability to grow on RM, scFOS, and their combinations. First, the growth curves for each strain were established, and the viable count of two representative strains was enumerated (CFU mL^{-1}) after 24 h of cultivation to evaluate the use of the PAS equation for its ability to select the best potential synbiotic combination. Subsequently, an alternative score was proposed, allowing a more accurate assessment of the growth ability of the probiotic strain on different fibres. Finally, the same score was recalculated for the four best-performing strains grown in minimal medium, and the benefits of using this minimal medium were discussed. Identifying the optimal combination of live microorganisms and substrates, with a focus on efficient prebiotic utilization by the selected probiotic strain, is essential for developing effective synergistic synbiotics. However, given that not all bacterial strains utilize prebiotic substrates equally, the variability in substrate processing highlights the importance of systematic evaluation methods to identify the most compatible strain–substrate combinations under diverse growth conditions and phases. Therefore, in this study, we introduced an alternative scoring method called the synbiotic potential score (SPS) as a more precise measure of substrate utilization and bacterial growth score for a superior approach to the identification of putative synergistic synbiotics.

Material and methods

Bacterial strains

Bifidobacterium longum APC 1472, *Bifidobacterium breve* JCM 7017, *Bifidobacterium bifidum* LMG13195, *and Bifidobacterium adolescentis* DSM 20083 were obtained from the APC Microbiome Culture Collection. *Bifidobacterium animalis* subsp. lactis ATCC 27536 and *Lacticaseibacillus rhamnosus* ATCC 7469a were obtained from the Moorepark Food Research Center (Teagasc, Fermoy, Co. Cork, Ireland). *Lactiplantibacillus plantarum* DSM 20205 and *Lacticaseibacillus paracasei* subsp. *paracasei* DSM 5622 were obtained from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH). All these strains are part of a healthy gut microbiome and are commonly found in human and murine faecal samples. Glycerol stocks of bacterial cultures were stored at −80°C in MRS broth (Thermo Fisher Scientific, 12 912 608) containing 0.05% L-cysteine HCl and 20% glycerol. All bacterial cultures were grown in an anaerobic hood (gas composition: $N_2 = 80\%$, $H_2 = 10\%$, $CO_2 = 10\%$).

Prebiotics and MRS media preparation

RM 97 (94% RM, 0.5% sugars, 1% digestible carbohydrates, and 4% water), RM 85 (87% RM, 1%, 8% digestible carbohydrates, and 4% water), and short chain fructooligosaccharides (scFOS preparations, (EUOLIGO® FOS) 95 (91% scFOS, 4% sugar, and 5% water) were obtained from Tate & Lyle PLC, in powder form. The R M preparations (PROMITOR® Soluble Fibre, Tate & Lyle, UK) consist of soluble corn fibres with a total carbohydrate content of ∼95%, dietary fibre contents of 85% and 94% respectively, and minimal free sugars (2% in RM 85; 0.5% in RM 97). The remaining carbohydrate fraction represents small amounts of digestible oligosaccharides (8% in RM 85; 0.5% in RM 97). scFOS is composed primarily of short-chain fructo-oligosaccharides (1-kestose, nystose, and 1F-fructofuranosylnystose) with glucose and fructose as monomeric sugars. MRS medium with prebiotics (per 100 mL) was prepared by dissolving MRS without glucose (Condalab, 1295) (3.52 g in 100 mL) containing 0.05% L-cysteine HCl in 80 mL of deionized water and autoclaved for 15 min at 121°C. Subsequently, 10% of each prebiotic solution was prepared in 20 mL of DI water and rendered sterile by filtration (pore size 0.2 μm) prior to its addition to previously autoclaved media without glucose to achieve a final concentration of 2%. DI water and 10% glucose solution were used as negative and positive controls, respectively.

Growth curves generation using 96-well plate reader

To assess the effect of specific prebiotics on the growth of selected bacterial strains, growth curves were generated using a portable plate reader (Cerillo Stratus Plate Reader; Fisher Scientific). Bacteria of interest were streaked onto MRS agar plates from stock cultures and incubated at 37°C for 24/48 h in an anaerobic hood until single visible colonies were observed. Subsequently, a tube containing 10 mL of MRS medium was inoculated with a single colony from the agar plate and incubated at 37°C in an anaerobic hood. After reaching turbidity (OD $>$ 0.8, equivalent to between 10^8 and 10^9 CFU mL^{-1}, depending on the strain), 0.01 mL of bacterial liquid culture (1% vol/vol) was transferred into 0.99 mL MRS broth containing no glucose, 2% glucose, or 2% prebiotic, and 200 μL of each combination was transferred into wells of a 96 well plate in triplicate. The 96-well plate was then placed in a portable plate reader, and the OD600 of each well was measured every 20 min for 24/48 h. The 96 well plate reader was stored in the anaerobic hood for the whole duration of the experiment, to ensure the anaerobic conditions.

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Bacterial enumeration

The strains of interest were streaked onto MRS agar plates from stock cultures and incubated at 37°C for 24/48 h in an anaerobic hood. Subsequently, a tube containing 10 mL MRS medium was inoculated with a single colony from an agar plate and incubated at 37°C in an anaerobic hood. After reaching the late exponential/early stationary phase (OD $>$ 0.8, equivalent to between 10^8 and 10^9 CFU mL^{-1}, depending on the strain), 0.1 mL of bacterial liquid culture (1% vol/vol) was transferred into MRS broth containing no glucose, 2% glucose, or 2% prebiotic and incubated for 24 h at 37°C under anaerobic conditions. The cultures were enumerated in CFU mL^{-1} on MRS agar plates at 0 and 24 h. A spot plate technique was used, in which 20 μL of each dilution was spotted and allowed to dry on a single plate. Each enumeration was performed in triplicates. The agar plates were then incubated for 24/48 h at 37°C in an anaerobic hood.

Growth curves in minimal MRS

To better assess the growth of probiotic strains on various prebiotics, a minimal version of MRS medium was used without interference from rich media. To achieve this, yeast and beef extracts were removed from the media, and the minimal version was called minMRS (composition per L: 2 g dipotassium phosphate, 10 g peptone, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 manganese sulfate, 1 g TWEEN 80, and 0.5 g L-cysteine HCl). For growth assessment using an automatic plate reader in minMRS, the overnight culture was first spun down at 4500 $\times$ *g* for 20 min at room temperature, and the pellet was reconstituted in PBS to avoid the effects of the ingredients from the rich media on the results. The experiment was conducted in the same manner as that for the rich MRS medium.

Statistical analysis

For statistical analysis, a *t*-test was performed with a confidence interval of 95%. Both analyses were performed using GraphPad Prism version 8.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Synbiotic potential score in MRS

When selecting suitable candidates for putative pre- and probiotic combinations, it is important to select strains that can utilize substrates to confer health benefits to the host. Therefore, the growth of different strains on substrates must be assessed. PAS is commonly used to evaluate the prebiotic potential of complex carbohydrate. It is calculated by comparing the growth of selected probiotic strains on the prebiotic to their growth on glucose using CFU mL^{-1} obtained at specific time points (e.g. 24 or 48 h), regardless of growth differences. Since its establishment in 2007, several studies have used this method to select the best probiotic combinations. However, because different strains exhibit different growth kinetics, we hypothesized that using a single enumeration time point could lead to biased conclusions and that using growth curves may be a more accurate representation of bacterial growth on different substrates.

To this end, the growth of eight putative probiotic strains on different dietary fibres was evaluated by measuring the optical density at 600 nm (OD_{600}). The strains were selected based on their potential to metabolize prebiotics of interest, as determined in previous studies. To achieve high throughput, they were grown in 96-well plates using selected prebiotic substrates (scFOS, RM, and their combination), and their growth kinetics were monitored over time to establish their growth curves (Fig. 1a–h). This approach enabled an approximate comparison of the efficiency of each strain in metabolising different fibres in a short time.

Among the tested prebiotics, scFOS supported the most robust bacterial growth. The highest OD_{600} values were recorded for *B. longum* APC 1472 (Fig. 1a), *B. adolescentis* DSM 20083 (Fig. 1b) and *L. plantarum* DSM 20205 (Fig. 1d) when they were grown on scFOS. This indicates that these strains readily utilize FOS as an energy source, consistent with previous reports of its high fermentability by bifidobacteria and lactobacilli (Kaplan and Hutkins 2000, Dong et al. 2024, Matera 2024). In contrast, growth on RM was minimal for all strains compared to growth on scFOS and could not be accurately assessed when scFOS data were present. They were presented side by side (Fig. S1) to allow comparison of bacterial performance in the presence and absence of RM. Although the observed increases in OD_{600} were modest, *B. longum* APC 1472, *B. adolescentis* DSM 20083, *B. animalis* subsp. lactis ATCC 27536, and *B. breve* JCM 7017 exhibited measurable growth on RM compared to media lacking glucose, indicating a limited but detectable capacity to utilize this fibre.

This limited growth was anticipated because RM is a branched, high-molecular-weight prebiotic ($\sim$2000 Da) that is only partially digestible ($\sim$10% based on composition) (Rösch et al. 2015). Both RM soluble fibres (85 and 97) differ in dietary fibre content, branching and linkage composition. In contrast, the scFOS preparation has a molecular weight of $\sim$600 Da and consists of linear, unbranched chains of fructose units linked by β-(2$\rightarrow$1) bonds with a terminal glucose unit (Campbell et al. 1997). The reduced utilization of RM by individual bacterial strains is consistent with the established knowledge that the collective activity of faecal microbiota, which contains a wide variety of carbohydrate-active enzymes (CAZymes), enables more efficient degradation of structurally complex carbohydrates (Anand et al. 2018). Without the synergistic action of multiple microbial species, the ability to metabolize such substrates is markedly diminished.

It is evident, that each strain exhibited distinct kinetics in reaching the stationary phase, followed by death, during which viable counts were taken. In addition, this time varied even between the strains grown on different prebiotics. For instance, *B. breve* required over 20 h to reach the exponential phase when grown on scFOS compared to only 10 h when grown on glucose (Fig. 1h).

To illustrate how enumeration time impacts PAS results, we selected two representative strains, *B. longum* APC 1472 and *B. animalis* subsp. *lactis* ATCC 27536, which differ markedly in growth rates. These strains were enumerated after 24 h of incubation in media containing different prebiotics. Our objective was to establish a rapid high-throughput screening method for identifying optimal synbiotic combinations. Focusing on two well-characterized strains with contrasting growth profiles allowed us to clearly demonstrate the capabilities of the method while minimizing variables, thereby ensuring a clear proof-of-concept.

Viable cell counts (VCCs) were expressed as CFUs per millilitre for *B. longum* and *B. animalis*. For *B. longum*, the difference in

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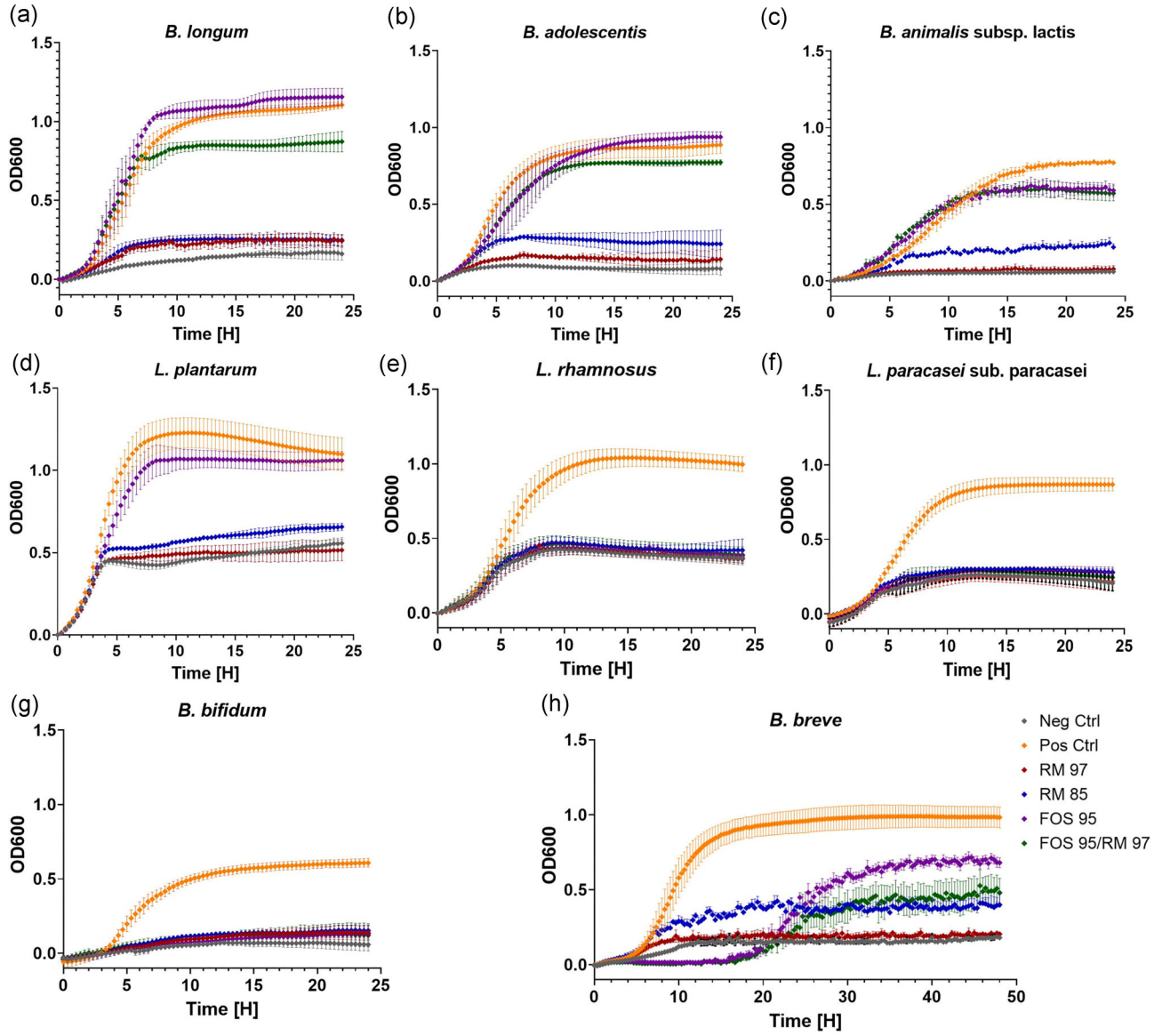


Figure 1 Growth curves of eight probiotic strains in MRS containing selected prebiotics. Glucose was used as a positive control (orange), and medium without a carbohydrate source was used as a negative control (gray). The data points of the readings were recorded every 20 min for 24/48 h. Data are presented as the mean ± SEM ($n = 3$).

CFU mL^{-1} between cultures grown without a carbohydrate source (negative control) and with glucose (positive control) was minimal (Fig. 2a). This result was inconsistent with the corresponding growth curve data (Fig. 1a), in which cultures grown on glucose reached an $OD_{600} > 1$, compared with ∼0.2 for the carbohydrate-free control. Moreover, the VCC profiles across the prebiotics differed substantially from those observed in the growth curves.

Based on OD_{600} measurements, *B. longum* APC 1472 exhibited maximal growth on glucose and scFOS, followed by the scFOS/RM 97 combination. In contrast, VCC data indicated the highest viable counts on scFOS/RM 97, while growth on glucose and scFOS was comparable to that on RM alone, patterns not reflected in the optical density data. A likely explanation for these discrepancies is that enumeration was performed after the cultures had entered the death phase, thereby underestimating the growth on certain substrates. Growth curve analysis confirmed that *B. longum* entered the stationary phase within 5–10 h on all tested substrates in 200 μL cultures. Although the time to reach the stationary phase would increase in larger volumes (e.g. 10 mL), the growth rate suggests that *B. longum* likely reached the stationary and subsequent death phases within 24 h.

In contrast, *B. animalis* displayed a slower growth rate, as indicated by its growth curves (Fig. 1a), reaching the stationary phase on all substrates in ∼10–15 h. For this strain, the VCC values (Fig. 1b) closely matched the maximum OD_{600} values from the growth curves (Fig. 2d). Both data types indicated optimal growth on glucose, followed by scFOS, scFOS/RM, RM 85, and RM 97.

These findings indicate that when using VCC to evaluate probiotic growth on prebiotics, the enumeration timing must be tailored to the growth kinetics of each strain–substrate combination. However, adjusting the enumeration time introduces additional experimental variability. To mitigate this, we recommend integrat-

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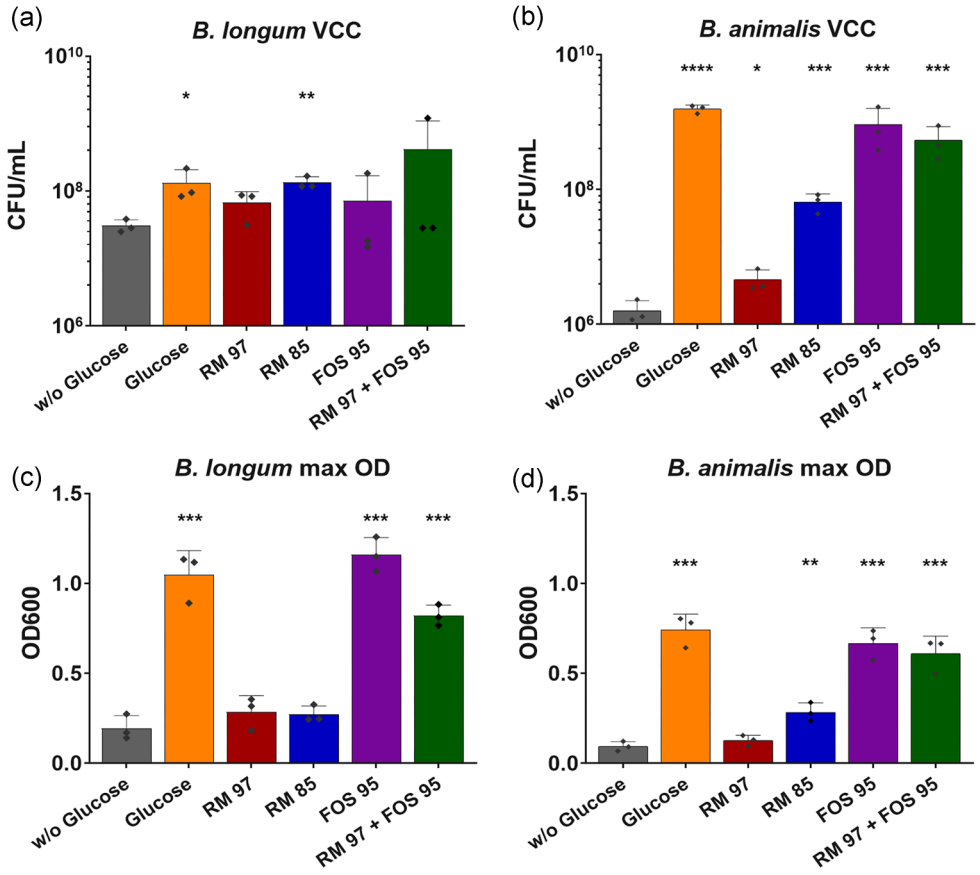


Figure 2 (a) Viable cell counts expressed as CFU mL^{-1} of *B. longum* grown in MRS with and w/o prebiotics for 24 hours; (b) Viable cell counts of *B. animalis subsp. lactis* grown in MRS with and w/o prebiotics for 24 hours; (c) Maximum OD based on growth curves of *B. longum* in MRS with and w/o prebiotics for 24 hours in a 96-well plate; (d) Maximum OD based on growth curves of *B. animalis subsp. lactis* grown in MRS with and w/o prebiotics for 24 hours in a 96-well plate. Data are presented as the mean ± SEM ($n = 3$).

ing growth curve data, particularly minimum and maximum OD_{600} values, to account for strain-specific growth dynamics.

Here, we propose an alternative scoring method to PAS, the SPS, which we present as a more precise method for assessing the growth and utilization of bacterial strains on selected fibres. Unlike PAS, which relies on growth comparison with glucose based on CFU mL^{-1} measurements at fixed time points (e.g. 24 or 48 h), SPS was derived from the minimum and maximum OD_{600} values obtained from growth curve experiments. This approach allows differences in growth characteristics between bacterial strains and prebiotic substrates to be taken into account. Although most strains used in this study reached the stationary phase within 24 h, *B. breve* grown on scFOS and scFOS/RM reached the stationary phase between 20 and 30 h (Fig. 1h).

Additionally, we included a negative control, represented by the OD600 value obtained from the medium without a carbohydrate source, to account for bacterial growth in the medium without a carbohydrate source. For instance, *L. plantarum* reached an OD600 of ~0.5 when grown on RM, which could lead to a high PAS for this prebiotic. However, compared to its growth in MRS without a carbohydrate source, this strain reached similar ODs without prebiotics (Fig. 1d). The MRS medium is optimized for lactobacilli, which may explain why it provides sufficient nutrients to support growth. Therefore, it is essential to account for baseline bacterial growth in the absence of a carbohydrate source, to distinguish strains capable of proliferating without prebiotic supplementation

In the original PAS formula, growth of the probiotic on a candidate prebiotic fibre was normalized to the growth of a commensal bacterium such as *E. coli*. This approach assumes that stimulation of *E. coli* indicates poor prebiotic selectivity. However, since certain *E. Coli* strains are the basis for commercially available probiotic products (Ukena et al. 2007, Beimfohr 2016, Sonnenborn 2016, Wassenaar 2016), using *E. coli* as a comparator could bias the interpretation. Therefore, this comparison was excluded in the development of the SPS metric.

The formula for SPS calculation established in this study is summarized in Equation 1 below.

$$\frac{(ODmax_{preb} - ODmin_{preb}) - (ODmax_{negCtrl} - ODmin_{negCtrl})}{(ODmax_{posCtrl} - ODmin_{posCtrl}) - (ODmax_{negCtrl} - ODmin_{negCtrl})}$$

Eq. 1 Updated equation to calculate Synbiotic Potential Score (SPS); $ODmax_{preb}$ – max OD600 reached on specific prebiotic, $ODmin_{preb}$ – min OD600 reached on specific prebiotic, $ODmax_{posCtrl}$ – max OD600 reached on glucose, $ODmin_{posCtrl}$ – min OD600 reached on glucose, $ODmax_{negCtrl}$ – max OD600 reached w/o carbohydrate source, $ODmin_{negCtrl}$ – min OD600 reached without carbohydrate source.

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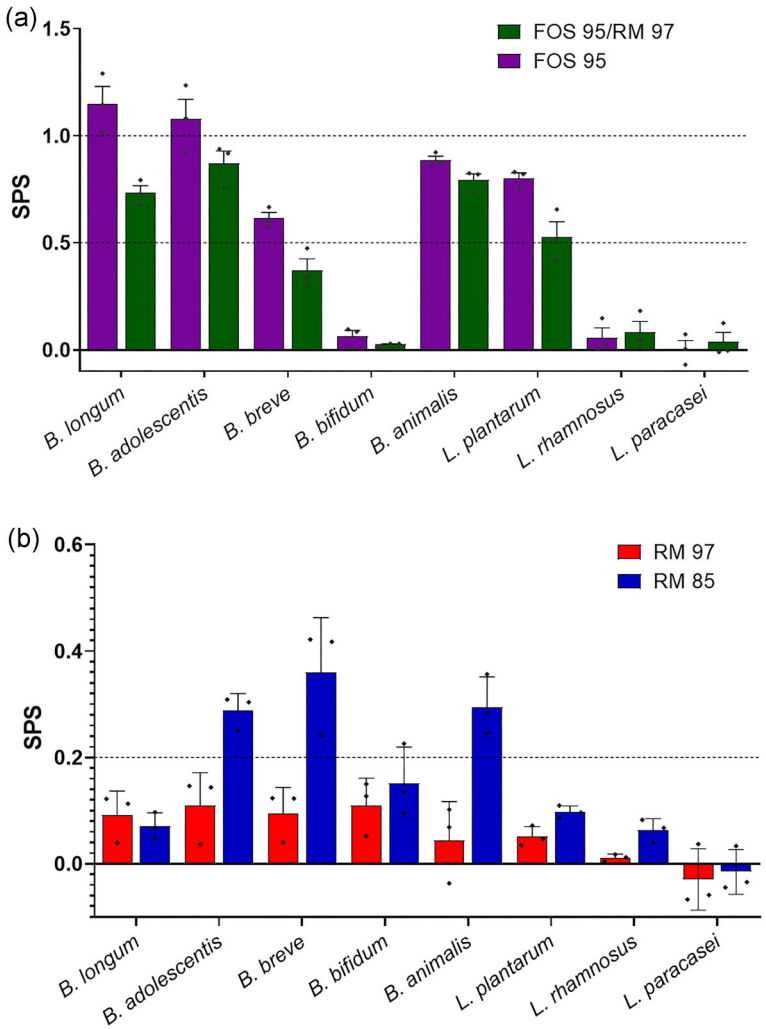


Figure 3 (a) SPS calculated for selected strains when grown on FOS and FOS/RM 97 combination; (b) SPS calculated for selected strains when grown on RM 97 and RM 85.

The SPS values obtained for the selected strains for each prebiotic were summarized (Fig. 3). As expected, based on the growth curves, SPS values were highest for strains grown on scFOS, with two strains (*B. longum* and *B. adolescentis*) having a score above 1, suggesting better growth on scFOS than on glucose. *Bifidobacterium animalis*, *L. plantarum,* and *B. breve* also scored high on scFOS, suggesting that these strains can utilize this glycan. *Bifidobacterium bifidum*, *L. rhamnosus,* and *L. paracasei* were unable to utilize scFOS efficiently and were therefore excluded from further experiments.

Regarding the growth on RM 97 and RM 85, as expected, the SPS scores were significantly lower than those on scFOS, ranging from 0 to ∼0.2 for RM 97 and 0 to ∼0.5 for RM 85. The higher SPS score for RM 85 compared to that of RM 97 is likely due to the higher concentration of sugars and digestible oligosaccharides and the lower level of branching. This suggests that RM 85 is more accessible for the growth of *B. adolescentis*, *B. breve*, and *B. animalis*.

This scoring approach can be used to compare the growth performance of different probiotic bacteria on various prebiotic substrates. Scores obtained from the same prebiotic substrate across different strains can be directly compared, and comparisons can likewise be made among distinct bacterial species and probiotic formulations. However, the substrates evaluated must already be established as prebiotics. The scoring system does not determine whether a given fibre is specifically metabolized by the microorganisms in question; rather, it assumes prebiotic functionality. This could be further extended, similar to PAS score, if a bacterial strain known not to have probiotic effects is utilized and the growth performances compared.

The highest SPS obtained on both, RM and scFOS was used to select of four strains for follow-up experiments. When considering only RM 97, *B. longum*, *B. adolescentis*, *B. breve*, and *B. bifidum* had the highest scores. However, the relatively good score for *B. bifidum* on RM was mostly driven by a poor growth of this strain on glucose. In general, it showed poor growth on the selected substrates, including scFOS. When combined with the SPS scFOS, *L. plantarum* emerged as another strong candidate which was finally selected instead of *B. bifidum*, along with *B. longum*, *B. adolescentis*, *B. breve.*

SPS in minimal MRS

Although the previous experiment revealed strain-specific differences in growth on various prebiotics, evaluating fibres such as

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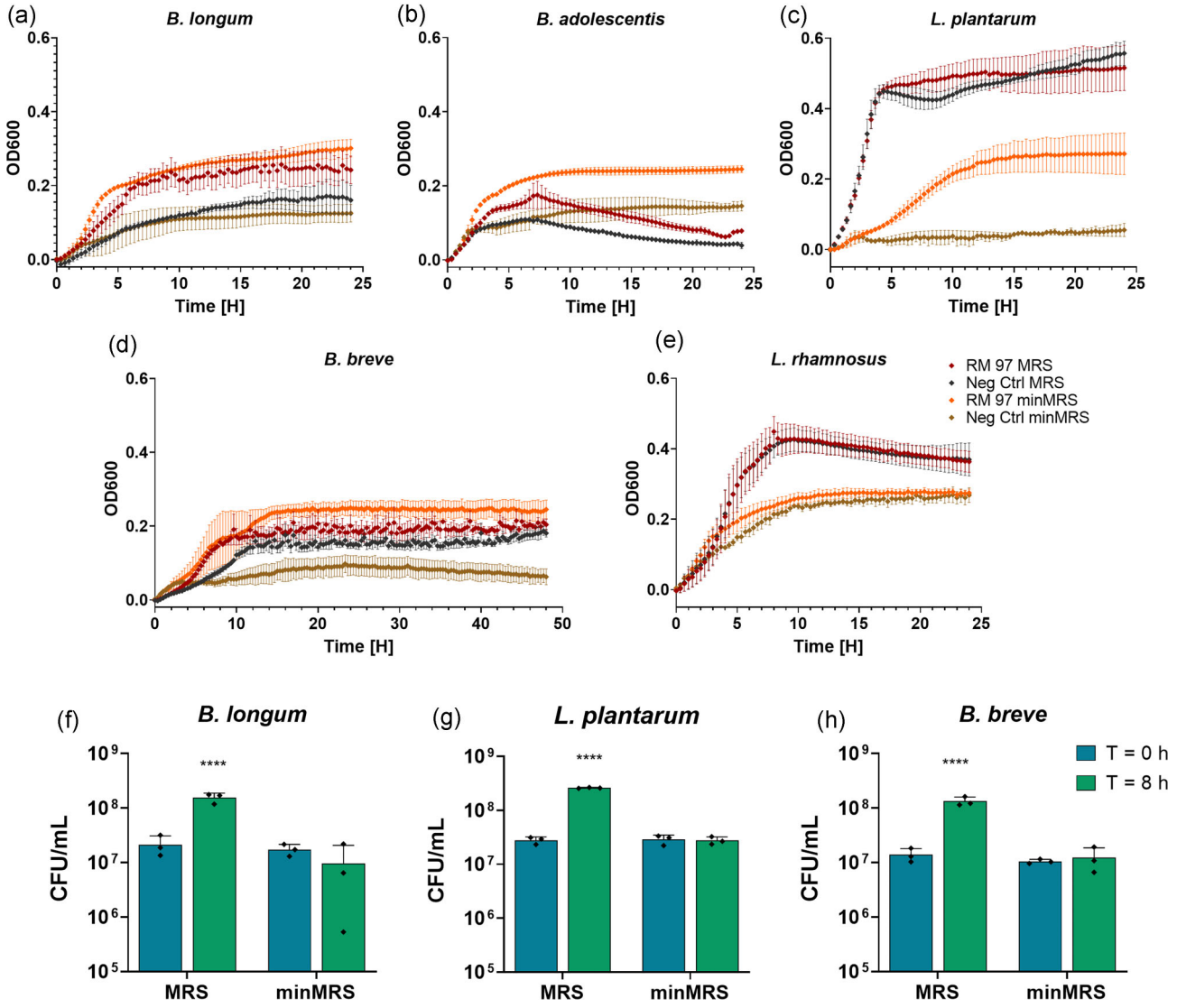


Figure 4 Growth curves with and without RM in MRS and minMRS media for (a) *B. longum*, (b) *B. adolescentis*, (c) *L. plantarum*, (d) *B. breve*, and (e) *L. rhamnosus*. Data are presented as the mean ± SEM ($n = 3$). VCC in full MRS and minMRS without prebiotics at T = 0 h and T = 8 h for (f) *B. longum*, (g) *L. plantarum*, and (h) *B. breve*. Data are presented as the mean ± SEM ($n = 3$).

RM, which are poorly metabolized by the individual strains, proved challenging. This limitation is likely attributable to the nutrient-rich composition of the MRS medium, which can sustain the growth of certain strains even in the absence of an added carbohydrate source. Based on the SPS calculations, four strains were selected for further investigation: *B. longum* APC 1472, *B. breve JCM 7017*, *L. plantarum* DSM 20205, and *B. adolescentis* DSM 20083. *L. rhamnosus* was included as a negative control. Growth curves were obtained under conditions similar to those of the previous experiment, except that overnight cultures were first centrifuged and resuspended in PBS to remove residual nutrients from the rich medium.

The rationale for this approach was to establish a nutritionally reduced medium capable of supporting bacterial viability while forcing the reliance on the supplemented prebiotic as the primary carbon source. This would enable a more accurate assessment of prebiotic utilization through growth. The growth of the four selected strains in minimum MRS (minMRS) supplemented with RM 97, scFOS 95, and RM 97/scFOS 95 was assessed (Fig. S2). The first notable difference between MRS and minMRS was that strains grown on glucose did not achieve OD600 comparable to that of cultures in full MRS. This outcome was expected, as the reduced nutrient availability in minMRS would limit bacterial biomass accumulation. However, the difference between growth in the presence of RM and the negative control was more pronounced in minMRS, highlighting the increased sensitivity of this medium for detecting prebiotic-dependent growth (Fig. 4a–e).

The growth profiles of the four selected strains showed minimal differences between the rich MRS medium supplemented with or without RM, consistent with previous observations. In contrast, replacing the base medium with minMRS revealed clear growth differences in the presence and absence of RM. Among the strains tested, *L. plantarum* exhibited the most pronounced OD600 increase with RM relative to the negative control, followed by *B. breve*, *B. adolescentis*, and *B. longum*. In contrast, *L. rhamnosus* displayed no change in the maximum OD600 between RM and the

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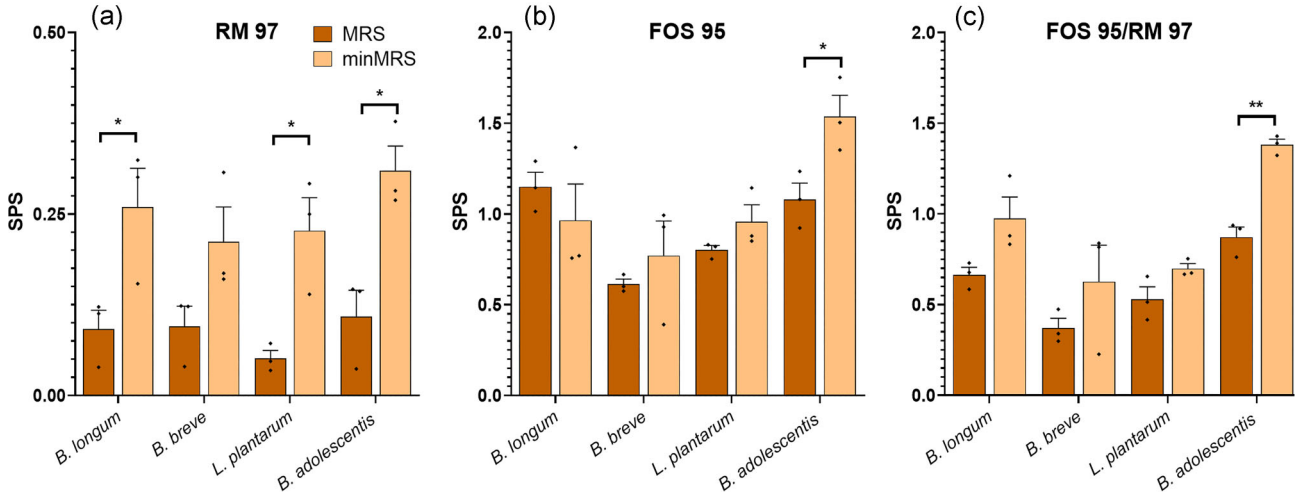


Figure 5 SPS calculated for selected strains grown in MRS and minMRS: (a) RM 97, (b) FOS 95, and (c) FOS 95/RM 97. Data are presented as the mean ± SEM ($n = 3$).

negative control, confirming its inability to utilize RM and supporting its exclusion from subsequent assays.

These findings emphasize the necessity of employing minimal media for prebiotic evaluation, as nutrient-rich media may hide prebiotic-dependent growth. To further evaluate the capacity of the selected strains to grow in the absence of carbohydrate supplementation, VCC in rich MRS and minMRS without prebiotics were determined (Fig. 4f–h). The strains were enumerated at 0 and 8 h. Based on the growth curves obtained from the microplate assay, we hypothesized that cultures in 10 mL volumes without carbohydrate supplementation would reach the stationary phase within 8 h. However, this estimate was made as part of a proof-of-concept study and was not independently validated.

All three tested strains exhibited a significant increase in VCC after 8 h of incubation in MRS medium. In contrast, when grown in minMRS, the VCC did not change. The strains remained viable but did not proliferate in the absence of added carbohydrate sources. These results demonstrate that minimal media allows prebiotic-driven growth to be distinguished more effectively from background growth supported by rich media components.

The revised SPS was calculated from the growth curves of the four probiotic strains in minMRS (Fig. 5) and compared with the scores previously obtained in rich MRS for each prebiotic. SPS values for RM were higher in minMRS than in MRS, indicating that certain strains metabolized RM only when alternative nutrients were limited. In contrast, when rich MRS was used, strains such as *L. plantarum* DSM 20205, *B. longum* APC 1472, and *L. rhamnosus* ATCC 7469a preferentially utilized carbon sources from the media for growth, regardless of the added carbohydrates. The most pronounced effect was observed for *L. plantarum*, which displayed enhanced growth on RM in minMRS medium. The scores obtained in the RM were significantly higher in minMRS than in rich MRS, suggesting that some bacterial strains could metabolize RM when other nutrients were not available.

For scFOS, the SPS values in minMRS were largely comparable to those in MRS, except for *B. adolescentis*, which exhibited significantly higher scores in minMRS (Fig. 5b). Similar trends were observed for the scFOS/RM combinations, with scFOS serving as the dominant substrate, again with *B. adolescentis* showing increased growth in minMRS.

Discussion

Our findings underscore the importance of employing minimal media to evaluate bacterial growth on complex fibres. This approach avoids the nutrient-driven bias inherent to rich media and enables more accurate identification of prebiotics, particularly those that are difficult to metabolize, such as RM.

Although minimal media based on MRS components have been used previously, the novelty of this study lies in the systematic integration of a defined minimal medium with high-throughput growth curve analysis and the SPS. This combined framework enables a more precise, scalable, and comparable assessment of prebiotic utilization, directly addressing a key gap in synbiotic formulation strategies. Applying SPS under minimal media conditions (minMRS) revealed important strain-specific differences that were not apparent in the rich MRS medium. Future research should investigate the combination of strains to evaluate the potential for enhanced metabolism of prebiotics, particularly RM. In addition, the area under the optical density (OD) growth curve (AUC) could be considered as an alternative to the OD_{max}–OD_{min} measurement in follow-up experiments for selected strains, as it integrates information across the entire growth trajectory and may provide additional insight into microbial growth dynamics across the lag, exponential, and stationary phases. However, within the context of the current SPS formulation, OD_{max}–OD_{min} was retained as it directly reflects the growth amplitude used in the calculation. Incorporating AUC would require modification of the formula to account for differences in the time required to reach stationary phase, a parameter that was not relevant for SPS.

Conclusion

Recent advances in prebiotic and synbiotic research have highlighted the importance of *in vitro* screening approaches to iden-

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tify effective strain–substrate combinations. In this study, we developed and applied the SPS as a quantitative approach to assess strain-specific prebiotic utilization. Unlike the traditional Prebiotic Activity Score (PAS), SPS incorporates full growth-curve data to provide a reproducible measure of substrate utilization. SPS applied under minimal media (minMRS) conditions revealed enhanced growth on RM and scFOS/RM combinations, distinguishing genuine prebiotic-driven growth from background proliferation. Among tested strains, *L. plantarum*, *B. breve*, *B. adolescentis*, and *B. longum* showed improved utilization of RM under minimal conditions, supporting the sensitivity of SPS to detect substrate-specific growth. We integrated SPS with minimal media to provide a reproducible, scalable method for high-throughput evaluation of strain–substrate interactions and selection of optimal prebiotic combinations. By accounting for growth kinetics, SPS in minimal media minimizes confounding factors and improves assay sensitivity. While this approach effectively identifies promising strain–substrate pairs *in vitro*, prebiotic utilization does not always reflect enhanced fitness or activity *in vivo*, underscoring the need for follow-up studies in more physiologically relevant systems. Future work should validate SPS-identified combinations in functional cellular, *ex vivo*, and ultimately *in vivo* models, thereby advancing a mechanistic framework for rational synbiotic selection.

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Author contributions

Luiza Adela Wasiewska (Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft), Lamiah Kareem (Investigation, Methodology), Ainhoa Linares (Investigation, Methodology), Eoin Gunnigle (Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing), Davide Risso (Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing), Ieva Laurie (Conceptualization, Funding acquisition, Methodology), Douwe Van Sinderen (Conceptualization), Gerard Clarke (Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing), Harriët Schellekens (Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing).

Supplementary material

Supplementary material is available at the *Journal of Applied Microbiology* online.

Conflict of interest

G.C. received honoraria from Janssen, Probi, Heel Pharmaceuticals, Ingelheim Boehringer and Apsen as an invited speaker, and research funding from Pharmavite, Reckitt, Tate and Lyle, Nestle, Fonterra and is or has been a paid consultant for Heel Pharmaceuticals, Bayer Healthcare, Yakult and Zentiva. This support did not influence or constrain the study design, collection, analysis, data interpretation, or writing of the manuscript. All other authors report no conflicts of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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