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Combinations of bacterial probiotics and yeast postbiotics influence fat deposition and growth in the nematode *C. elegans*

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ABSTRACT

Background: Probiotics are live microorganisms with intended benefits on human health including obesity. As a small and fast-growing whole organism model, *Caenorhabditis elegans* has been used to assess the health effects of probiotics where mechanisms can be assessed through available genetic tools. Results from *C. elegans* can provide data on the effect of specific probiotic strains and combinations with prebiotics and postbiotics on health-related physiology to inform selections of interventions for further study. We hypothesized that specific combinations with prebiotics and postbiotics could both speed up worm development and reduce fat deposition, suggesting they allow for more effective nutrient utilization.

Methods: Here we expose *C. elegans* to the ABB S20 strain of *Lactiplantibacillus plantarum* in combination with different prebiotics and postbiotics. We then measure how these affect growth and development speed as well as fat deposition by measuring the time until the appearance of progeny and measuring Oil Red O staining respectively.

Results: Our results show that the combination of probiotic *L. plantarum* ABB S20 plus the postbiotic inactive yeasts *K. marxianus* ABB S8 and *S. boulardii* ABB S3 resulted in fast growth and reduced fat deposition compared to *L. plantarum* ABB S20 alone.

Conclusion: These results demonstrate the usefulness of *C. elegans* as a model to efficiently screen though combinations of probiotics, prebiotics and postbiotics to find those that are candidates to help with effective nutrition use and therefore weight management.

1. Introduction

Obesity, which can be a result of imbalance between energy intake and consumption, unhealthy lifestyle, and genetic variability, is a prevailing human health problem that is associated with type 2 diabetes, cancer and other health conditions. Research into the role of the intestinal microbiome in the development of obesity has shown that there are several genetic, metabolic, and inflammatory pathophysiological mechanisms involved [1–3]. The microbial composition of the intestine can be a significant factor for the development of obesity as obese and non-obese patients have been found to have distinct microbial compositions in their intestines [1]. Modulating these compositions has been shown, in animal and human studies, to affect energy harvest from the diet and energy storage in the host and can result in weight loss [1,4,5].

Such modulation of intestinal microbiome has been achieved using probiotic bacteria or prebiotics which are non-digestible ingredients that promote the growth of beneficial bacteria in the intestine [6,7]. While probiotics and prebiotics have been the subject of many studies in which their beneficial effect has been demonstrated, synbiotics, which are various combinations of probiotics, prebiotics and postbiotics, are attracting an increasing amount of interest. Postbiotics include cell-free

Abbreviations: ADUs, Analogue Digital Units; CGC, Caenorhabditis Genetics Center; DM, Defined Media; DM -a/a, Defined Media without amino acids; LB, Luria-Bertani; MCT, Medium Chain Triglyceride; MRS, DeMan, Rogosa and Sharpe; ORO, Oil Red O.

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extracts of bacteria or other microorganisms such as yeast, nonviable microorganisms and other microbial components [6–8].

The ever-increasing number of combinations of the above ingredients requires a cost and time-effective system that can test for their beneficial effects on weight management in whole organism physiology to inform selections for further study. *Caenorhabditis elegans* is a free-living nematode that feeds on bacteria in soil and rotting vegetation. Through extensive achievements by thousands of research groups across the world *C. elegans* has become a powerful model system to study diverse aspects of biology such as ageing, neuronal function, and obesity among others and is also a useful tool for drug discovery and preclinical screens [9]. For example, beneficial effects have been shown to translate from *C. elegans* to rodents in a study by Ryu et al. [10] where urolithin A was shown to induce mitophagy and prolonged lifespan in the worms but also increased muscle function in rodents. Many basic physiological processes, signaling pathways and genes affecting fat metabolism are conserved from *C. elegans* to humans [2,11,12]. In addition, powerful genetics along with its anatomy have made *C. elegans* an effective system to study accumulation of macromolecules under different conditions [2, 13–15]. The worms feed on the benign OP50 strain of *Escherichia coli* under lab conditions but can feed on and grow on several bacterial species including probiotic bacteria. *C. elegans* has also been used as a model for studying the effects of probiotics and postbiotics [7,16]. Fat staining of fixed worm populations grown on different bacterial strains or postbiotics can be used to quantify the effects of different interventions on fat deposition [14,15,17]. With a lifecycle of only three days at 24°C, and the ability to easily synchronize worms, rates of growth and development can be monitored carefully.

Here we study ABB S20, a live strain of *Lactiplantibacillus plantarum* (previously called *Lactobacillus plantarum*). *L. plantarum* is well known to have probiotic effects as it can help reduce cholesterol in adults [18], reduce obesity in mice [19] and in *C. elegans*, was shown to extend longevity, delay ageing and stimulate innate immunity pathways [20]. In this study, we measure both the speed of *C. elegans* developmental growth and the deposition of fat compared to worms grown on the standard strain *E. coli* OP50. We hypothesized that specific combinations of *L. plantarum* ABB S20 with prebiotics and postbiotics could speed up worm development and reduce fat deposition, suggesting they allow for more effective nutrient utilization. Using *L. plantarum* ABB S20 as a food source, we performed a screen of combinations with prebiotics and postbiotics. Understanding how these combinations affect *C. elegans* development and fat deposition may provide mechanistic insight by which probiotics and yeast products can be selected to assist human weight management.

2. Materials and methods

2.1. Probiotics, prebiotics and postbiotics

The probiotics and postbiotics used in this study are described in Table 1. The bacterial strains were cultured in their respective media from the dry powders, streaked out onto plates to visually observe any possible contamination. Glycerol stocks were made from single colonies and stored at −80°C. OP50 was grown at 37°C in LB (Luria-Bertani) broth under aerobic conditions. *L. plantarum* ABB S20 was grown at 37°C in MRS (DeMan, Rogosa and Sharpe) media under anaerobic conditions.

2.2. Preparation of assay agar plates

Defined agar media [21] without amino acids (DM -a/a) was prepared by adding agar at 2 % and NaCl at 0.3 % to distilled water and autoclaving at 121°C for 20 minutes. Once media had cooled to 55°C, the following solutions were added: 1 M Magnesium Sulphate at 0.1 %, 1 M Potassium Phosphate buffer pH 6 at 2.5 %, Cholesterol stock solution (5 mg/ml, dissolved in ethanol) at 0.1 %, Trace metals stock solution at 0.02 %, 100 μM Vitamin B12 stock solution at 0.01 %, Uracil stock solution (2 mg/ml dissolved in distilled water and filter sterilized) at 0.2 %. Each 6 cm petri dish was filled with 15 ml of media.

Table 1
List of Probiotic, prebiotic and postbiotic ingredients used in this study.

Name	Species/ingredient	Type of strain/ ingredient	Source
OP50	*Escherichia coli*	Live bacteria	CGC
ABB S20	*Lactiplantibacillus plantarum*	Live bacteria	AB Biotek HNH
ABB S3	*Saccharomyces boulardii*	Heat Inactivated yeast, Freeze dried	AB Biotek HNH
ABB S8	*Kluyveromyces marxianus*	Heat Inactivated yeast, Freeze dried	AB Biotek HNH
ABB I5	Prebiotic dextran from *Weisella cibaria*	Prebiotic fiber, powder	AB Biotek HNH
ABB I4	Medium Chain Triglyceride (MCT) oil	Food supplement	AB Biotek HNH
NCIMB 30370	*Lactobacillus gasseri*	Live bacteria	Isolated from commercially available sources
HA4597/ AF036	*Hafnia alvei*	Live bacteria	Isolated from commercially available sources
CECT 8145	*Bifidobacterium animalis subsp. lactis*	Live bacteria	Isolated from commercially available sources
DGCC B420	*Bifidobacterium animalis subsp. lactis*	Live bacteria	Isolated from commercially available sources

On the day of adding the bacteria to the assay plates, the cultures were centrifuged at 3000 rpm for 5 minutes and washed two times in M9 buffer [3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 liter. Sterilize by autoclaving]. The final pellet was resuspended in equal volume of M9 buffer [22]. The inactive yeasts, *S. boulardii* ABB S3, *K. marxianus* ABB S8 and the prebiotic fiber ABB I5 were resuspended in M9 at 20 mg/ml on the day of adding to plates. The bacterial and yeast resuspension were mixed in appropriate amounts along with M9. 100 μl of the mix was plated on each DM -a/a plate, allowed to dry and kept at 24°C overnight. The final concentrations of the inactive yeasts were 1 mg/ml, the prebiotic fiber ABB I5 was 6 mg/ml, and ABB S19 was 1 %.

2.3. C. elegans strains, maintenance and preparation

C. elegans strains, N2 (Wild Type) and SS104 *glp-4(bn2)* (temperature-sensitive sterile) and the bacterial strain OP50 were obtained from CGC (CGC, University of Minnesota, Minneapolis, MN, USA). Worms were grown on defined media at 15°C with OP50. To obtain worms for the growth and development assay, 20 gravid N2 worms from unstarved cultures were placed on 9 cm plates. Egg lays were done for two days after which the gravid worms were removed. Worms for the fat staining assay were obtained similarly with 20 gravid SS104 worms being placed on 9 cm plates for egg lays. Four days after setting up egg lays, the plates with SS104 worms were transferred to 24°C to induce sterility.

2.4. Semi-quantitative growth and development assays

Four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 h and any other observations were noted. Representative images were taken each day using a stereo dissecting scope (Leica MZ10F). The depletion of the bacterial lawn was also scored by assessing if there was bacteria remaining on the plates. We collected data for 10 days because of the slower growth rate seen on

some combinations compared to growth on the standard OP50 bacteria.

2.5. Fat staining assays

DM -a/a plates were prepared with the combinations of bacteria and yeasts as specified in the results. SS104 *glp-4(bn2)* worms that were sterile after being shifted to 24°C to prevent full germline formation the previous day were picked as L4 larvae on to each experimental plate. 6 plates of 50 worms were picked to make a total of ~ 300 worms per condition. The plates were kept at 24°C for 4 days to allow development under exposure to different symbiotic combinations. Adult worms were washed off the plates with 1.5 ml M9-T (M9 + 0.1 % Tween 20) and used for the fat staining assay.

Quantifying fat deposition was performed using the Oil Red O (ORO) staining protocol adapted from Escorcia et al. [23]. ORO working solution was made by diluting ORO stock solution (0.5 % Oil Red O solution in isopropanol, SIGMA) 3:2–60 % isopropanol and mixed overnight on a shaker at 24°C. The working solution was filtered through a 0.22 μm PES filter before use. Wash from all the plates for a particular condition was collected in one culture tube. Worms were allowed to settle, and the turbid supernatant was carefully removed. Worms were transferred into a 1.5 ml Eppendorf tube for subsequent processing. Worms were washed three times with 1 ml M9-T by centrifuging at 500 rpm for one minute to settle the worms. The supernatant was removed till 100 μl remains. Worms were fixed by adding 600 μl, 40 % isopropanol and rocking at room temperature for 3 minutes, settled by spinning at 500 rpm, 30 sec. and the fix removed so that 100 μl remains. 600 μl ORO working solution and the worms were mixed well in ORO solution. The tubes were rocked at 40 rpm on a shaker at 24°C for 2 h, spun at 500 rpm for 1 min and the staining solution removed until 100 μl remains. 600 μl of M9-T was added and the worms washed by rocking the tubes at 40 rpm on a shaker at 24°C for 30 min. Worms were settled by spinning 500 rpm for 1 min and supernatant removed so that 50 μl remains. The tubes were kept overnight at 4°C. The next day, 5 μl of worm suspension was added to a clean slide with an agarose pad (2 % agarose in water, made fresh) and covered with a 22 mm×50 mm coverslip.

Images of stained worms were taken on a stereo dissecting microscope (Leica MZ10F) at 0.8x magnification with the same illumination, camera settings and exposure for all conditions.

Measurement of Oil Red O intensity as analogue digital units (ADUs) was performed using Image J as follows: The images were converted to RGB and using the blue channel the outlines of the worms were selected and the mean intensity was measured. The background mean intensity was measured and absorbance was calculated as A=1-(worm intensity/background intensity). A minimum of 90 worms per treatment were imaged.

2.6. Statistical analyses

The statistical analyses for the Oil Red O fat staining assays were performed using GraphPad Prizm 9. One-way ANOVA tests were used to compare the mean ADUs of the various treatments assuming the populations follow a Gaussian distribution and have the same standard deviation. Statistical significance was reported as * $p<0.1$, ** $p<0.01$, *** $p<0.001$ or **** $p<0.0001$.

2.7. Patents

These findings have been included in a European Patent application with number EP23383159.

3. Results

3.1. Effect on growth and development

The first semi-quantitative growth and development assay showed that worms grown on *L. plantarum* ABB S20 had delayed development compared to worms grown on the standard *E. coli* (OP50) strain (Fig. 1). While on *E. coli* OP50, worm populations needed 72 h to reach the 2nd generation (G2), the populations grown on *L. plantarum* ABB S20 required 108 h. Combining *L. plantarum* ABB S20 with *K. marxianus* ABB S8 reduced this time to 84 h. Combining *L. plantarum* ABB S20 with *K. marxianus* ABB S8 plus prebiotic fiber ABB I5 or *K. marxianus* ABB S8 plus MCT oil ABB I4 reduced the time to G2 to 96 h. Finally, combining *L. plantarum* ABB S20 with *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 reduced the time to 72 h, which was the same as the *E. coli* OP50 control.

The second growth and development assay included a selection of commercially available probiotics (Fig. 2). These were compared with *L. plantarum* ABB S20 and the results showed that they all reduced the time required to reach G2. On *H. alvei* HA4597 and *L. gasseri* NCIMB 30370, the worms needed 60 h while on *B. lactis* DGCC B420 or *B. lactis* CECT 8145, they needed 66 and 72 h respectively.

The third growth and development assay included combinations using *S. boulardii* ABB S3 and *K. marxianus* ABB S8 for which the first experiment and the fat staining experiment showed promising results (Fig. 3). Here, worms fed *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 needed 72 h to reach G2, while worms fed on *L. plantarum* ABB S20 plus *S. boulardii* ABB S3 required 96 h.

3.2. Effect on fat deposition

To validate and standardize the Oil Red O protocol for measuring fat deposition, we first used starved (for 24 h) and un-starved populations of sterile SS104 day-4 adult worms (Fig. S1) and confirmed that starvation decreased fat staining ($p=0.0012$). Based on the results, we chose the optimal staining time and settings for imaging and quantifying the amount of fat deposition.

Using these conditions, we conducted the first fat staining assay with sterile SS104 day-4 adult worms that had been exposed to the different lawn combinations from L4 to Day 4 adulthood. Here, we found that the *L. plantarum* ABB S20 and *E. coli* OP50 conditions did not differ significantly with respect to fat deposition (Fig. 4). Combinations of *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 and *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus MCT oil ABB I4 resulted in a statistically significant lower fat deposition compared to the *L. plantarum* ABB S20 control ($p<0.0001$ and $p=0.0011$ respectively). The other two combinations, *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 and *L. plantarum* ABB S20 plus prebiotic fiber ABB I5 did not lead to a statistically significant difference compared to the *L. plantarum* ABB S20 control.

Based on the results of the first fat staining assay and the respective growth and development results, we performed a fat staining experiment including the commercially available samples and the most promising ABB combination: *L. plantarum ABB S20* plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 (Fig. 5). The results showed that of the commercial samples, only *B. lactis* DGCC B420 led to a statistically significant reduction of fat deposition, compared to the *L. plantarum* ABB S20 control ($p<0.0001$). *H. alvei* HA4597, *B. lactis* CECT 8145 and *L. gasseri* NCIMB 30370 did not have a statistically significant effect. *L. plantarum* ABB S20 plus *S. boulardii* ABB S3 and *L. plantarum ABB S20* plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 did appear to reduce the fat content but the result did not reach statistical significance.

4. Discussion

The aim of this study was to develop a strategy for using *C. elegans* to efficiently screen combinations of probiotics, prebiotics and postbiotics

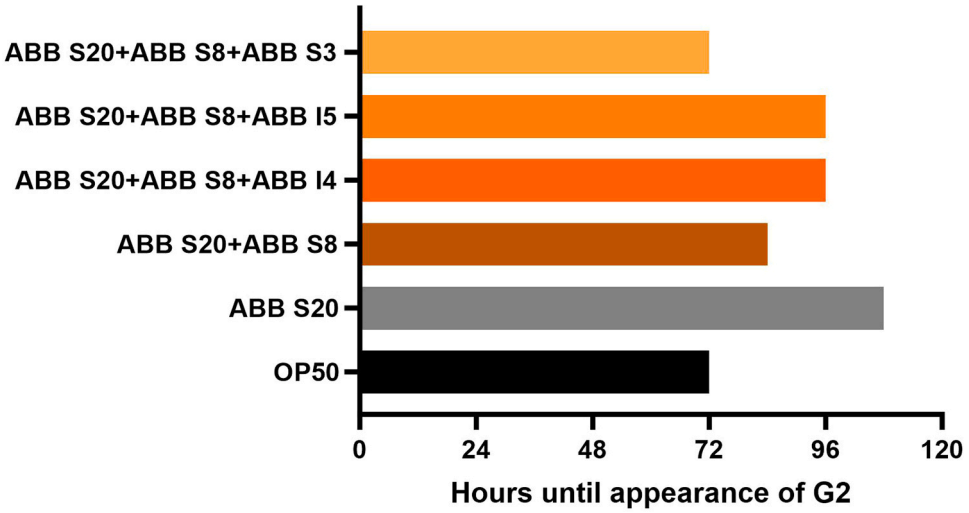

Fig. 1. Time (hours) until the appearance of the second generation (G2) of progeny in worm populations fed on *E. coli* OP50, *L. plantarum* ABB S20 or combinations with *L. plantarum ABB S20*. To measure the effect of probiotics, prebiotics and postbiotics on developmental growth, four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 hours. *L. plantarum* ABB S20 slowed down growth compared to *E. coli* OP50 while the combination of *L. plantarum* ABB S20 with *K. marxianus* ABB S8 plus *S. boulardii* ABB S3, *K. marxianus* ABB S8 plus ABBI5, *K. marxianus* ABB S8 plus MCT oil ABB I4 or *K. marxianus* ABB S8, resulted in faster growth compared to worms fed *L. plantarum* ABB S20 alone.

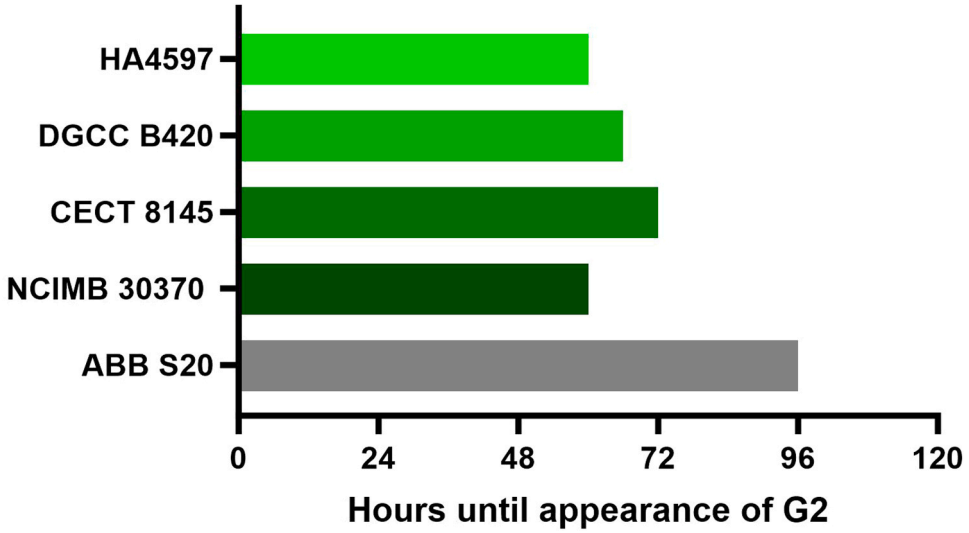

Fig. 2. Time (hours) until the appearance of the second generation (G2) of progeny in worm populations fed on *L. plantarum* ABB S20 or commercially available samples. To measure the effect of probiotics, prebiotics and postbiotics on developmental growth, four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 h. All the commercially available samples, *H. alvei* HA4597, *B. lactis* DGCC B420, *B. lactis* CECT 81145, and *L. gasseri* NCIMB 30370, resulted in faster growth compared to worms on *L. plantarum* ABB S20.

intended to have a beneficial effect on health and protect against obesity. Previous studies have used fat deposition staining to assess the effects of probiotics on *C. elegans* energy metabolism [24]. The drawback of this approach in isolation is that reduced fat deposition could also be the result of nutritional deficiency rather than improved energy metabolism. Therefore, we also measured the effect of our treatments on the speed of worm growth and development as a measure of effective nutrition. We hypothesized that the ideal combination of these ingredients will result in less body fat but without a negative impact on growth and development. Indeed, our results showed that it possible to lower fat deposition without affecting growth and nutrition and that this approach can inform decisions when selecting combinations of pro-, pre- and postbiotics for further study.

In laboratory conditions, *C. elegans* typically grow on *E. coli* OP50 [22], and it is well known that their growth and development is strongly affected when different bacterial strains are used [25]. To investigate the effect of the ingredients we wanted to test, we first sought to establish the baseline growth and development time of *C. elegans* when growing on *L. plantarum* ABB S20 (Fig. 1). Establishing *L. plantarum* ABB S20 as a suitable food source was important not only because of the probiotic effects of *L. plantarum* [18–20], but also because the worms are not able to directly consume the prebiotic fiber or yeast postbiotics that we wanted to test due to their size. Thus, we had to be sure that *L. plantarum* ABB S20 was a sufficient source of nutrition. However, we hypothesized that small sections of the postbiotic and prebiotic products (<2 μm) will be ingested directly by the worms along with molecules derived from these products, either directly, or indirectly via the bacteria, which can also take up important compounds [26]. It has already been shown that

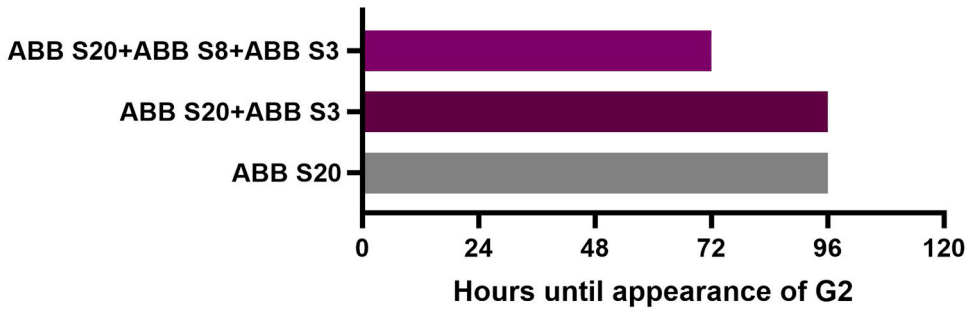

Fig. 3. Time (hours) until the appearance of the second generation (G2) of progeny in worm populations fed on *L. plantarum* ABB S20 or combinations with *L. plantarum* ABB S20. To measure the effect of probiotics, prebiotics and postbiotics on developmental growth, four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 h. Addition of *S. boulardii* ABB S3 to *L. plantarum* ABB S20 did not affect the growth of the worms but the addition of *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 resulted in faster growth compared to worms fed *L. plantarum* ABB S20 alone.

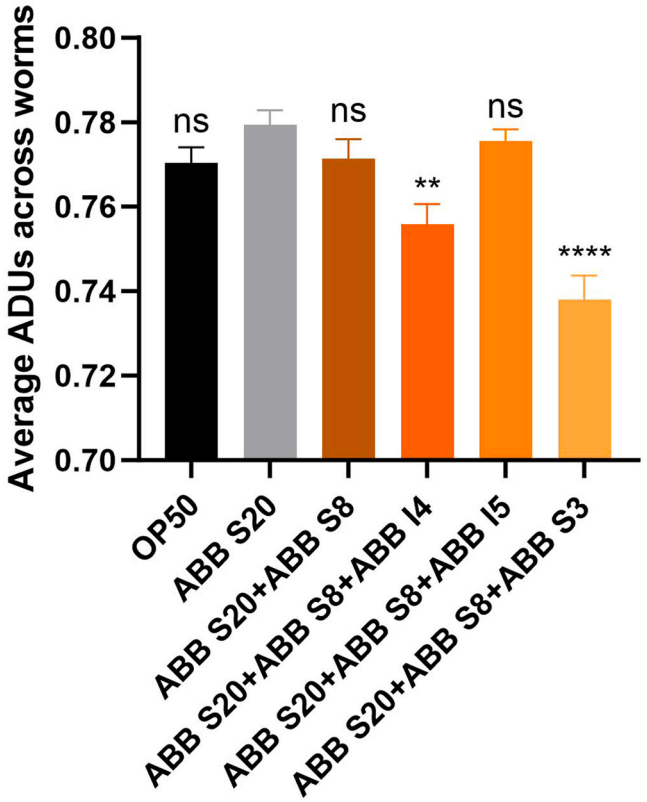

Fig. 4. Fat staining assay with worms fed *E. coli* (OP50), *L. plantarum* ABB S20 and combinations with *L. plantarum* ABB S20. To measure the effect of probiotics, prebiotics and postbiotics on fat deposition, SS104 *glp-4(bn2)* worms that were sterile after being shifted to 24°C to prevent full germline formation the previous day were picked as L4 larvae on to each experimental plate and were kept at 24°C for 4 days to allow development under exposure to different symbiotic combinations. Worms were collected and stained with Oil Red O and imaged. Measurement of Oil Red O intensity as analogue digital units (ADUs) was performed using Image J. Compared with *L. plantarum* ABB S20, two conditions, *L. plantarum ABB S20* plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 and *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 + MCT oil ABB I4, led to a statistically significant reduction in fat deposition ($p<0.0001$ and $p=0.0011$ respectively). The ADUs were compared using ANOVA (GraphPad Prizm). ** $p<0.01$. **** $p<0.0001$.

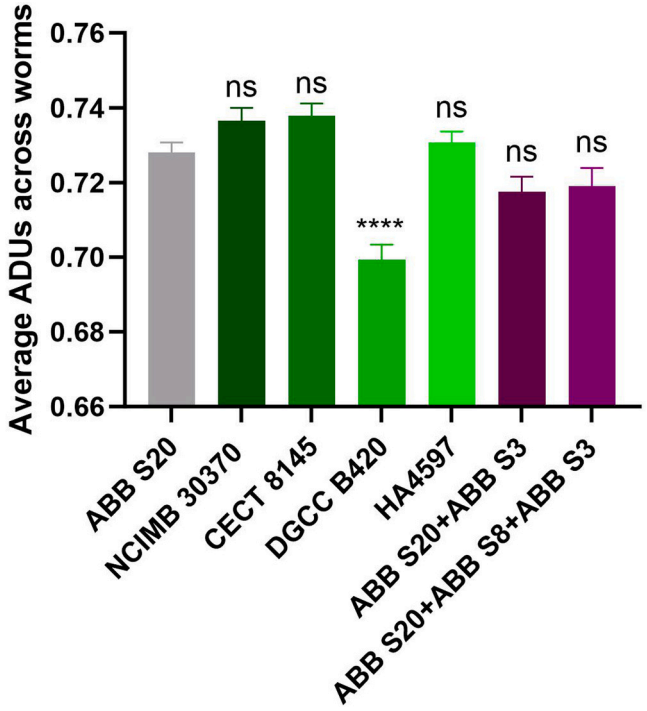

Fig. 5. Fat staining assay with worms fed *L. plantarum* ABB S20, commercially available samples and combinations with *L. plantarum* ABB S20. To measure the effect of probiotics, prebiotics and postbiotics on fat deposition, SS104 *glp-4 (bn2)* worms that were sterile after being shifted to 24°C to prevent full germline formation the previous day were picked as L4 larvae on to each experimental plate and were kept at 24°C for 4 days to allow development under exposure to different symbiotic combinations. Worms were collected and stained with Oil Red O and imaged. Measurement of Oil Red O intensity as analogue digital units (ADUs) was performed using Image J. Compared with *L. plantarum* ABB S20, only feeding with *B. lactis* DGCC B420, led to a statistically significant reduction in fat deposition ($p<0.0001$). The ADUs were compared using ANOVA (GraphPad Prizm). **** $p<0.0001$.

altered bacterial metabolism can impact *C. elegans* development and lifespan [21].

In this study we have demonstrated for the first time that these products influence worm physiology in combination with a probiotic strain. It has been shown that *S. boulardii* can be effective in improving nutrient uptake in humans by promoting the expression of digestive enzymes [27], while *K. marxianus* can reduce cholesterol levels in hypercholesterolemic subjects [28]. Prebiotic fiber ABB I5 and MCT oil ABB I4 were included because there are many studies concerning the beneficial effects of such ingredients in metabolic health [5,6,29–31]. These ingredients reduced the time for *C. elegans* to grow and develop

compared to *L. plantarum* ABB S20 alone (Fig. 1).

We next considered if prebiotic and postbiotic combinations with *L. plantarum* ABB S20 also affected the ability of the worms to deposit body fat. For these experiments we used strain SS104 *glp-4(bn2),* which is temperature-sensitive sterile, to prevent full germline formation that would interfere with the quantification of body fat. Worms fed only on *L. plantarum* ABB S20 deposited a similar amount of fat compared to worms fed on *E. coli* OP50 (Fig. 4) suggesting that the worms are not limited by energy production, but growth and development is possibly limited by a deficiency in other nutrients. The inclusion of prebiotics and postbiotics was able to affect the amount of fat that the worms stored with the combination of the inactivated yeasts *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 leading to the most significant reduction (Fig. 4). This result also suggests that the ingredients that cannot be digested by *C. elegans* can have an indirect effect through the bacteria that is ingested as we hypothesized. This conclusion is fits the current model that postbiotics function via effects on the gut microbiome [32].

This finding was particularly interesting because it demonstrated that the amount of fat deposition did not predict the speed of growth because the same combination of ingredients, *K. marxianus* ABB S8 plus *S. boulardii* ABB S3, combined both the fastest growth speed as well as the lowest fat deposition (Fig. 6). On the other hand, *L. plantarum* ABB S20 on its own resulted in the slowest growth and highest fat deposition. Thus, there are conditions in which growth can be optimized without an increase in body fat. As far as we know this approach is unique as no other studies of probiotics using *C. elegans* have combined these two measurements.

Based on this observation, we decided to include in our next experiments a selection of commercially available probiotic food supplements, that are intended to reduce obesity in humans, as a comparison with *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3. DGCC B420 is a strain of *Bifidobacterium lactis* for which there is evidence from *in vitro*, preclinical, and clinical studies often in combination with polydextrose. It has been shown to act through multiple signaling pathways to control weight gain and improve epithelial integrity in the gut [33]. CECT 8145, another *B. lactis* strain, has been shown to maintain its functional activities in a heat-treated form and reduce fat deposition in *C. elegans* while improving production of short-chain fatty acids [24]. Importantly, the positive effect of this strain seen in *C. elegans* was also demonstrated in clinical trials where it was found to have a beneficial effect on anthropometric adiposity biomarkers, particularly in women [34] as well as on abdominal adiposity and insulin levels in patients with Prader-Willi syndrome, without affecting total fat mass [35]. HA4597, a strain of *Hafnia alvei,* has not been tested using *C. elegans* but it has been shown, in a clinical trial, to help reduce obesity in overweight patients [36]. NCIMB 30370, a *Lactobacillus gasseri* strain, also had a positive effect on fat reduction in a clinical trial [37] and a different strain of *L. gasseri* was shown to promote longevity in *C. elegans* by increasing oxidative stress resistance and stimulating innate immune response signaling [38].

We found that growing *C. elegans* on *B. lactis* DGCC B420 resulted in increased growth and development speed as well as significantly lower fat deposition (compared to the *L. plantarum* ABB S20 control) (Fig. 2 **&** **5**). Faster development, compared to a control, suggests that nutrients are more readily available but if there is also higher fat deposition, then the effect is not desirable. On the other hand, looking at fat deposition alone is not sufficient as low-fat deposition may just be a result of nutritional deficiency or even toxicity. For this reason, our approach was designed to investigate the relationship between fat deposition and the utilization of nutrients for growth and development.

Finally, both the *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 combination and the commercial product *B. lactis* DGCC B420 resulted in fast growth and lower fat deposition (Fig. 7). It is noteworthy that neither *K. marxianus* ABB S8 nor *S. boulardii* ABB S3 reduce fat deposition when included individually, suggesting a synergistic interaction between these inactive yeasts when combined. Beneficial effects of individual prebiotic bacteria have been shown before and *C. elegans* has been used as a tool for screens. Feeding *C. elegans* with the probiotic bacteria *Lacticaseibacillus rhamnosus* HA-114 can restore disrupted lipid homeostasis and energy balance through mitochondrial β-oxidation resulting in suppression of neurodegeneration [16]. Also, a study that used *C. elegans* for a preclinical screen of 78 lactic acid bacteria strains identified Lactobacillus rhamnosus CNCM I-3690 that protected against oxidative stress and prolonged the worm's lifespan. In the same study it was shown that the bacteria strain also reduced inflammation in a murine model of colitis [39]. Our results further demonstrate the usefulness of *C. elegans* as a model to efficiently screen though food supplements that can help combat obesity, and in

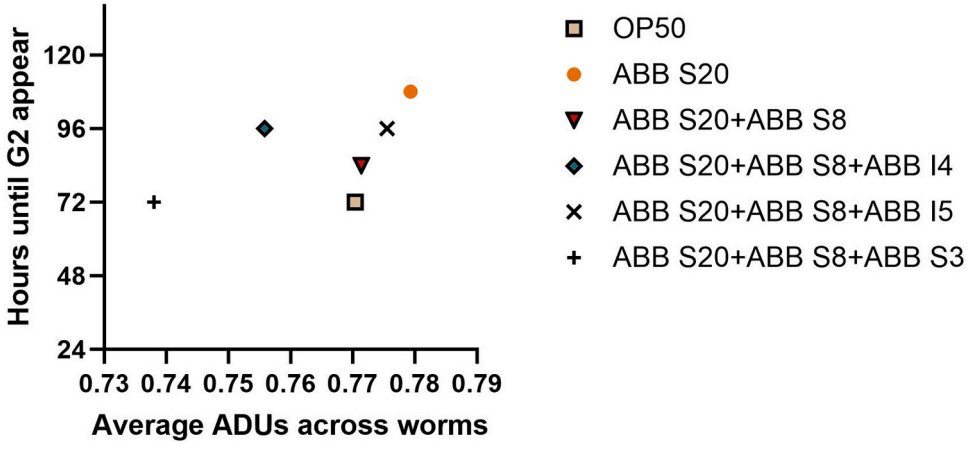

Fig. 6. Hours until the appearance of G2 in combination with average ADUs from the first set of experiments (*E. coli* OP50, *L. plantarum* ABB S20 and combinations with *L. plantarum* ABB S20). To measure the effect on developmental growth, four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 h. To measure the effect on fat deposition, SS104 *glp-4(bn2)* worms that were sterile after being shifted to 24°C to prevent full germline formation the previous day were picked as L4 larvae on to each experimental plate and were kept at 24°C for 4 days to allow development under exposure to different symbiotic combinations. Worms were collected and stained with Oil Red O and imaged. Measurement of Oil Red O intensity as analogue digital units (ADUs) was performed using Image J. The data from the two experiments was plotted to show the conditions that combined the fastest growth with the lowest fat deposition. The condition with the most beneficial effect according to these criteria was *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3.

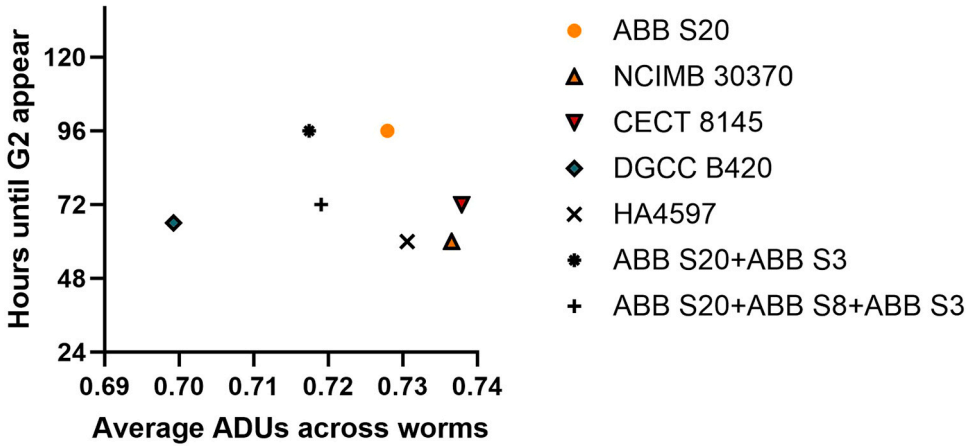

Fig. 7. Hours until the appearance of G2 in combination with average ADUs from the first set of experiments (*L. plantarum* ABB S20, commercially available samples and combinations with *L. plantarum* ABB S20). To measure the effect on developmental growth, four L4 stage larvae of wild type worms (N2 strain) were added to each of 3 plates for each combination and kept at 24°C. Worm progeny production and growth were monitored by eye every day for 10 days. The time for appearance of the second generation (G2) of both eggs and L1 larvae was estimated to the nearest 6 h. To measure the effect on fat deposition, SS104 *glp-4(bn2)* worms that were sterile after being shifted to 24°C to prevent full germline formation the previous day were picked as L4 larvae on to each experimental plate and were kept at 24°C for 4 days to allow development under exposure to different symbiotic combinations. Worms were collected and stained with Oil Red O and imaged. Measurement of Oil Red O intensity as analogue digital units (ADUs) was performed using Image J. The data from the two experiments was plotted to show the conditions that combined the fastest growth with the lowest fat deposition. Here, the condition with the most beneficial effect according to these criteria was *B. lactis* DGCC B420.

particular, the power of studying multiple combinations.

Fat metabolism involves diverse metabolic pathways and genes such as *spb-1, nhr-49, aak-2, tub-1, cebp-2* when mutated, affect fat metabolism in *C. elegans*. The mammalian homologs also affect fat metabolism, indicating conservation between worms and mammals [11,14]. Future work on the combinations found here could involve the use of genetic mutants, gene expression profiling and analysis of fatty acid composition to help elucidate the underlying mechanisms of fat reduction. This approach can help identify conserved mechanisms in humans but also lead to larger and more targeted screens with a higher likelihood of succeeding in more complex systems.

5. Conclusions

Understanding how these combinations affect *C. elegans* health and physiology may provide mechanistic insights by which probiotics and yeast postbiotics can be selected to assist human weight management. More importantly, *C. elegans* provides a fast and easy system to identify compounds, drugs or natural extracts on metabolic conditions such as fat deposition that are likely to have a positive outcome in clinical trials. Our finding of the *L. plantarum* ABB S20 plus *K. marxianus* ABB S8 plus *S. boulardii* ABB S3 combination and our validation of the commercial product *B. lactis* DGCC B420 as effective in reducing fat accumulation without causing a nutrient deficiency demonstrates the value of the system and paves the way for further studies.

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CRediT authorship contribution statement

David Weinkove: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Carlos de Lecea:** Conceptualization. **Jordi Cuñé:** Conceptualization. **Maria Tintoré:** Conceptualization. **Sushmita Maitra:** Writing – review & editing, Investigation, Conceptualization. **Michael Fasseas:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

Maria Tintoré, Jordi Cuñé and Carlos de Lecea are employees of AB Biotek Human Nutrition and Health. David Weinkove is a co-founder and shareholder of Magnitude Biosciences Ltd.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phanu.2024.100404.

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