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Study of the growth kinetics of probiotic strain *Lactobacillus helveticus* MTCC 5463 in the presence of different prebiotics

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Abstract
The study aimed at analyzing the growth characteristics of the probiotic strain *Lactobacillus helveticus* MTCC 5463 (V3) in the presence of different prebiotics. Prebiotics like Inulin, Lactulose and Fructo-oligosaccharide (FOS) was used in the study @ 2%. Prebiotic Index, growth rate, generation time (doubling time) was determined in the presence of each prebiotic. The results revealed that prebiotic index of FOS was significantly higher than all the prebiotics tested. The strain showed lower generation time and higher growth rate in the presence of FOS.

Keywords: *Lactobacillus helveticus* MTCC 5463, prebiotic, FOS, prebiotic index, growth rate, generation time

1. Introduction
Prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." The International Scientific Association of Probiotics and Prebiotics (ISAPP) gave a new definition for prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al.* (2017) [1]. Globally, the prebiotic market size is expected to grow from 6.05 billion US$ in 2021 with a CAGR of 14.9% between the year 2022 to 2030. The increase in the demand for prebiotics in dairy industry is due to the health benefits attributed upon its consumption (Grand view Research, (2021) [2].
The utilization of the prebiotic substance by the probiotic organism in the synergistic synbiotic combination can lead to the production of metabolites which is later metabolized by the residing beneficial microflora in the gut (Gomez *et al.* (2022) [3]. Boger *et al.* (2018) [4] studied the growth of individual probiotic bacteria in the presence of short chain inulin having Degree of Polymerization (DP) 2 to 40. They found that *Lactobacillus salivarius* W57 could only utilize the fraction of the inulin having DP 3 and 5 and there was no much growth of the organism in the presence of short chain inulin. Whereas, *Lactobacillus paracasei* subsp. *paracasei* W20 fully utilized all the fractions of the short chain inulin showing a luxurious growth. Davari *et al.* (2019) [5] reported that the *Bifidobacterium spp* can effectively utilize lactulose, starch and fructans. He also stated that inulin which has DP ≤ 60 was utilized only by few *Lactobacillus spp* whereas FOS having DP ≤ 10 was utilized by wide number of organisms. Fuhren *et al.* (2020) [6] studied the strain specific carbohydrate utilization of 77 *Lactobacillus plantarum* species. The test results revealed that, only a single strain LP 900 showed effective utilization of inulin and FOS. None of the other strains tested could fully utilize inulin and FOS.
In this background the current study is planned to evaluate the efficiency of the probiotic strain *Lactobacillus helveticus* MTCC 5463 in utilizing various prebiotics and to come up with the best synbiotic combination.

2. Materials and Methods
2.1 Bacterial strains
The probiotic strain *Lactobacillus helveticus* MTCC 5463 was taken from the Culture Collection of Dairy Microbiology Department, SMC College of Dairy Science, Kamdhenu University, Anand, Gujarat, India.

2.2 Prebiotics
Prebiotics used in the study were Inulin, Fructo-oligosaccharide (FOS) and Lactulose. Inulin and FOS were supplied by Gujarat Enterprise Ltd, Ahmedabad, Gujarat, India. Lactulose used was "Duphalac" by Abbott India Pvt. Ltd., Mumbai, India. Prebiotic solution (10%) was prepared by dissolving 10g of prebiotic in 100mL distilled water. The content was filter sterilized using 0.22µm membrane filter (Millex® - HV, MERK, Ireland) and stored in sterile glass bottles.

2.3 Prebiotic Index
Sterile de Man-Rogosa-Sharpe (MRS) and Glucose free MRS (GFMRS) broth were prepared in a conical flask. Prebiotics were added in MRS and GFMRS broths @ 2% and was mixed well. MRS broth without prebiotics was considered as the control. Broths were inoculated with active culture of V3 @ 2% and was incubated at 37±1 °C till 72h. In between time interval of 24 h, appropriate serial dilutions were made and the probiotic count was determined by pour plate method using MRS agar after incubation at 37±1 °C/48-72h. The colonies counted were expressed in CFU/mL. Various treatments used in the study include; Control (C): V3 + MRS, T_1: V3 + inulin +MRS, T_2: V3 + inulin + GFMRS, T_3: V3 + FOS + MRS, T_4: V3 + FOS + GFMRS, T_5: V3 + lactulose + MRS, T_6: V3 + lactulose + GFMRS.
The prebiotic index (PI) was determined confining to the method by (Palframan *et al.* (2003) [7]. The prebiotic index was calculated according Eq. (1);

$$\text{Prebiotic index (PI)} = \frac{\text{CFU of probiotic strain in the presence of prebiotic}}{\text{CFU of probiotic strain without prebiotic}} \quad (1)$$

2.4 Growth kinetics
Growth kinetics of the strain was investigated during the fermentation, both in the absence of prebiotics (control) and in their presence at a time interval of twelve hours. Optical density (OD) was measured using spectrophotometer at 600 nm wavelength. The maximum specific speed for the growth or growth rate (µmax) was calculated during the exponential growth phase using Eq. (2):

$$\mu\text{max} = \frac{1}{t2-t1} * \ln(x2/x1) \quad (2)$$

were, x2 and x1 represents the OD at 12 h (t2) and 0 h (t1) respectively.
Generation time (tg) or Doubling time was determined using Eq. (3):

$$tg = \ln 2 / \mu max \quad (3)$$

2.5 Statistical analysis
All the analysis was made using the statistical method, completely randomized design.

3. Results and Discussion
3.1 Prebiotic Index
Prebiotic index is defined as the ratio of growth of probiotic in the presence of prebiotic to growth of probiotic in a control carbohydrate substance. PI more than 1 indicates that the tested prebiotic substance has a positive influence on the probiotic growth and if the value is less than 1, it indicates that the tested prebiotic has a low effectiveness towards the growth of probiotic strain (Palframan *et al.* (2003) [7]. The prebiotic index was determined for evaluating the effectiveness of probiotic strain in utilizing the prebiotics.

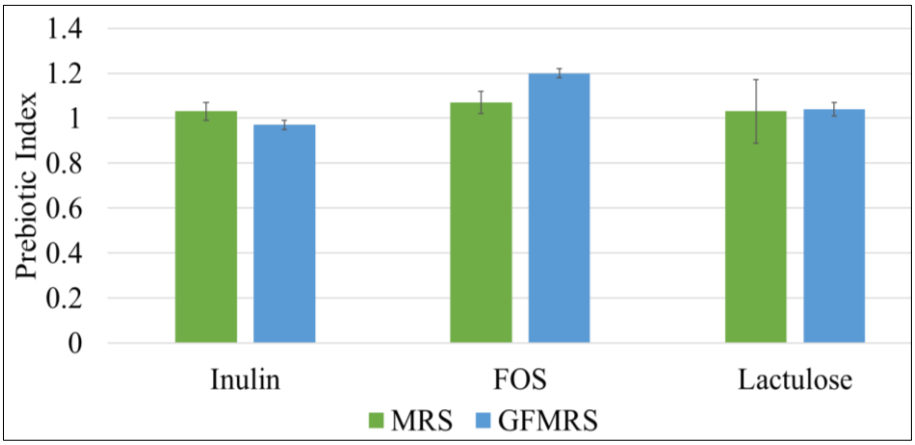

Fig 1: Prebiotic Index of different prebiotics in MRS and GFMRS+FOS media.

Our results (Fig. 1) showed that the mean prebiotic index of FOS (1.13) was significantly (p<0.05) higher than prebiotic index of inulin (1.00) and lactulose (1.03) after 48 h of incubation. PI of inulin and lactulose was not significantly different from each other. The results showed that the utilization of FOS by V3 was significantly better than the utilization of other prebiotics. Between the media (MRS and GFMRS), no significant difference in the PI was found statistically. Except for FOS, inulin and lactulose does not show a significant difference between the prebiotic index in two different media. In case of FOS, PI in GFMRS (1.20) was significantly (p<0.05) higher than in MRS (1.07). Irrespective of the growth media, whether FOS was given solely or in combination with other sugar like glucose, the strain *Lactobacillus helveticus* MTCC 5463 effectively utilized it.
Gonzalez *et al.* (2019) [8] reported highest prebiotic index for oligomate 55 (7.22) with the strain *Lactobacillus rhamnosus* GG. Kaewarsar *et al.* (2023) [9] reported that inulin (1.04) had a significantly (p<0.05) higher PI than FOS (0.86) and GOS (0.84) with probiotic cultures *Lacticaseibacillus rhamnosus* HII117 and *Bifidobacterium animalis subsp. lactis.* Pancham *et al.* (2023) [10] studied the prebiotic index of FOS and glucosamine (GS) with strain *Lactobacillus acidophilus* MTCC 10307. The PI of FOS was reported to be 2.8 at 3% concentration after the incubation period of 48 h.

3.2 Growth Kinetics

The average growth rate of V3 (Fig. 2) ranged from 0.02 h^{-1} to 0.27 h^{-1}. In case of control, the growth rate was 0.19 h^{-1}. In the presence of inulin, the growth rate in T_1 was 0.15 h^{-1} which was significantly (p<0.05) higher than in T_2 (0.02 h^{-1}). In the presence of FOS, growth rate in T_4 (0.27 h^{-1}) was significantly higher than in T_3 (0.15 h^{-1}). In the presence of lactulose, growth rate of V3 in T_5 (0.20 h^{-1}) was significantly higher than in T_6 (0.03 h^{-1}). While comparing the growth rate of V3 among all the treatments, T_4 (0.27 h^{-1}) was showing higher growth rate which was significantly (p<0.05) higher than all other treatments. The growth rates in control (0.19 h^{-1}) as well as in treatments T_5 (0.20 h^{-1}), T1 (0.15 h^{-1}) and T3 (0.15 h^{-1}) were found to be at par with each other. This may be because of the presence of glucose in the MRS medium, which is readily available for the growth of the strain.

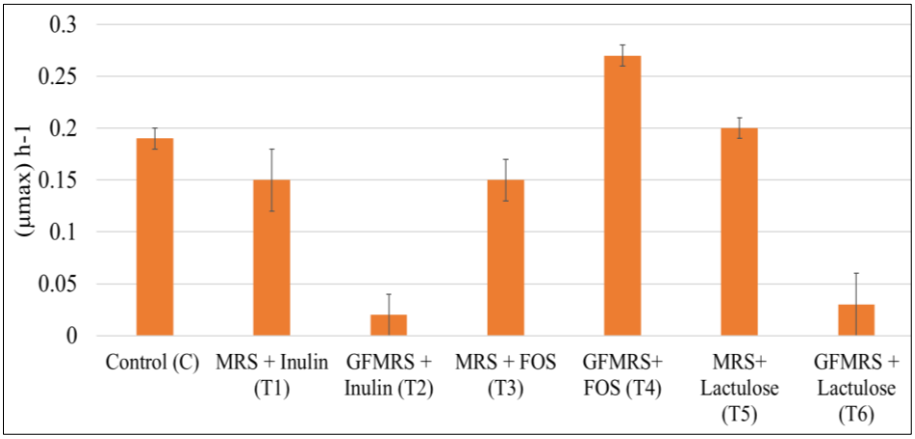

Fig 2: Growth rate of V3 in the presence of different prebiotics.

The average doubling time or generation time of V3 (Fig. 3) ranged from 2.00 h to 4.59 h. In case of control, the doubling time was found to be 2.34 h. In the presence of inulin as prebiotic, T_1 (2.57 h) had significantly (p<0.05) lesser doubling time than in T_2 (4.59 h). In the presence of FOS, T_4 (2.00 h) had significantly (p<0.05) lesser doubling time than T_3 (2.56 h). The doubling time of V3 in the presence of lactulose in T_5 (2.28 h) was significantly (p<0.05) lower than in T_6 (4.17 h). Among all the treatments, the doubling time of V3 was found to be significantly (p<0.05) lesser in T_4 (2.00 h).

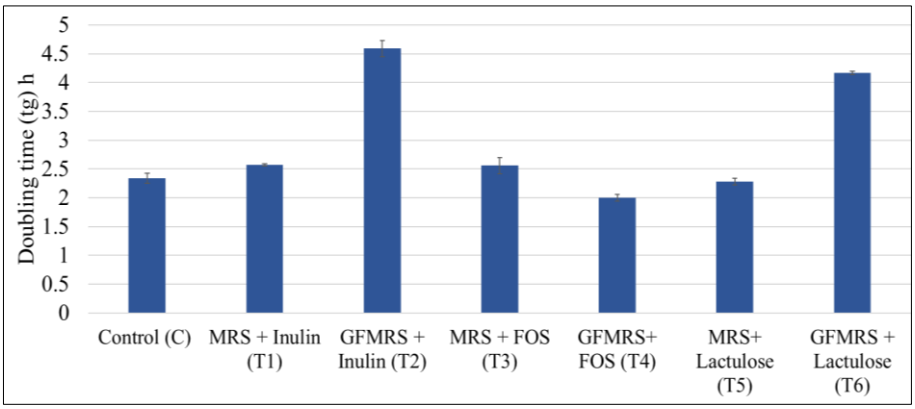

Fig 3: Generation time / doubling time of V3 in the presence of different prebiotics.

Farinha *et al.* (2015) [11] studied the growth kinetics of *Lactococcus lactis subsp. lactis* CECT 4434 in the presence of inulin, FOS, and poly dextrose (PD). The results showed that the specific speed for growth was 0.13, 0.48, 0.94, and 1.01 h^{-1} for glucose (control), FOS, PD, and inulin respectively. Inulin showed lower generation time of 0.68 h which was followed by PD (0.75 h), FOS (1.42 h), and control (2.66 h). Nagpal and Kaur (2011) [12] determined the doubling time of five probiotic strains *viz L. plantarum* M5, *L. plantarum* C2, *L. plantarum* Ch1, *L. casei* L1, and *L. helveticus* L3 in the presence of prebiotics like inulin, oligofructose, lactulose, raftilose, honey and glucose (control). They found that the doubling time of all cultures was lesser than the control. The lowest doubling time of 5.2 h was reported in the presence of inulin for the strain *L. plantarum* M5. The strain *L. helveticus* L3 showed highest doubling time of 9.6 h in the presence of honey. Kaewarsar *et al.* (2023) [9] reported that the strain *Bifidobacterium animalis subsp. Lactis* showed significantly higher growth rate (0.023 h^{-1}) with FOS than glucose (0.009 h^{-1}), inulin (0.010 h^{-1}) and GOS (0.008 h^{-1}).

4. Conclusion

Inulin, FOS and lactulose was imparting a prebiotic effect on the probiotic strain V3. Among these, higher prebiotic index was obtained with FOS. *Lactobacillus helveticus* MTCC 5463 exhibited higher growth rate and lower generation time in the presence of FOS. Hence, it can be concluded that FOS as prebiotic and probiotic *Lactobacillus helveticus* MTCC 5463 can be considered in the development of a synbiotic combination. Further *in vitro* and *in vivo* studies are warranted for in-depth understanding of the bio-functional properties of this synbiotic.

5. Acknowledgment
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