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NOVEL ASSESSMENT OF SYNERGISTIC STIMULATORY EFFECT OF PREBIOTIC CHITOOLIGOSACCHARIDE AND SOME COMMERCIAL PREBIOTICS ON THE PROBIOTIC GROWTH: A PRELIMINARY STUDY

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ABSTRACT

This study investigates the possible synergistic stimulatory effects of prebiotic chitooligosaccharide and some commercial prebiotics on probiotic growth. Different combination of chitooligosaccharide with inulin, fructooligosaccharide, and lactulose were prepared and their ability to stimulate the growth of probiotic strains was evaluated. Following Chou-Talalay method, the combination index was calculated and used for synergistic assessment. The data showed that most of the prebiotics combinations showed synergism with different magnitudes depending on the prebiotic type, concentration and the probiotic strain employed in the study. It was indicated from the combination index that the combination between chitooligosaccharide and fructooligosaccharide has higher synergism compared to the combination of chitooligosaccharide with lactulose or inulin. Additionally, as the concentration of prebiotics increased, the synergistic effect increased. Synergism was also affected by the probiotic strain employed, since a higher synergism was shown with *Bifidobacterium lactis* when compared to *Lactobacillus helveticus*. It was concluded that combining chitooligosaccharide with commercial prebiotics produced synergistic effect on the probiotic proliferation. The synergism was further dependent on the type and concentration of the combined prebiotic and on the probiotic strain used in the study.

Keywords: Chitooligosaccharide, *Bifidobacterium lactis*, Combination index, Inulin, Synergism

INTRODUCTION

The human digestive system is colonized by a complex ecosystem of 100 trillion microorganisms that are essential for gastrointestinal homeostasis (**Ouwehand and Vaughan, 2006**). Several environmental factors including smoking (**Biedermann *et al.*, 2013**), unbalanced diet **(Scott et al., 2013)**, and lack of physical activity (**Clarke *et al.*, 2014**) have been evaluated for the qualitative and quantitative composition of that ecosystem. The disturbance in the composition of these microorganisms has been correlated to some gastrointestinal diseases in addition to cardiovascular and emotional disorders. Moreover, restoring the balance of intestinal microbiota has been indicated to improve the health of those patients and prevent complications (**Bailey and Cryan, 2017; Feng *et al.*, 2018**). Hence, it is always desirable to maintain and restore the balance of intestinal microbiota to maintain beneficial health effects.

Probiotic administration has been known for long time to be the main agents influencing the composition of intestinal microbiota. They are defined according to the Food and Agriculture Organization of the United Nations and World Health Organization (**Report FAO/WHO, 2001**) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Czinn and Blanchard, 2009 reported that the minimum daily intake of probiotics to show its health benefits was 10^8-10^{10} cfu (cells-colony forming unit). In the last decade, a large number of food products and beverages enriched by probiotics (such as *Lactobacilli* and *Bifidobacteria*) are commercially produced (**Sanders *et al.*, 2019**).

On the other hand, prebiotics are food components that selectively boost the microbiota proliferation in the intestinal tract. The International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2016 revised the prebiotic definition to 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' (**Gibson *et al.*, 2017**). Although this definition expanded the utilization of different substrates including inorganic and organic substances as prebiotics, non-digestible carbohydrates including galactans and fructans are known as the most widely applied substrates. Several clinical studies demonstrated the positive modulation of the intestinal microbiota by prebiotic consumption. **Drakoularakou *et al.*, 2010** and **Hasle *et al.*, 2017** reported a significant reduction in the incidence of travelers' diarrhea by the consumption of β-galacto-oligosaccharides. In addition, inulin has been found to be efficient in the reduction of adults and children constipation (**Yurrita *et al.*, 2014; Closa-Monasterolo *et al.*, 2017**). Another study by **Staudacher & Whelan., 2016** and **Vulevic *et al.*, 2018** reported a significant improvement in some gastrointestinal symptoms of irritable bowel syndrome by the consumption of β-galacto-oligosaccharides.

The synergistic benefits of combining the prebiotics with the probiotic bacteria (synbiotics) in food supplement to boost the intestinal microbiota has been explored by various scientific literatures **(Femia *et al.*, 2002; Crittenden *et al.*, 2003; Bartosch *et al.*, 2005; Cook *et al.*, 2014; Westfall *et al.*, 2018; Fayed *et al.*, 2018; Maftei., 2019**). The consumption of *Bifidobacterium animalis* subsp. *Lactis* 420 in combination with Litesse Ultra™ polydextrose led to modulation of the gut microbiota that may support the improvement in the function of the gut barrier and the obesity-related markers (**Hibberd *et al.*, 2019**). **Shimizu *et al.*, 2018**, also indicated the modulation of the gut microbiota after the consumption of *Bifidobacterium breve* strain Yakult, *Lactobacillus casei* with galacto-oligosaccharides suggesting its preventive effect on the incidence of enteritis and ventilator-associated pneumonia in patients with sepsis.

As the consumed probiotic strain must compete with an already established microbiota, it was found that the synergistic effect of combining probiotic bacteria and prebiotics can be related to the ability of the probiotic bacteria to adapt to the prebiotic substrate prior to consumption which provides an advantage for synbiotic consumption over the consumption of probiotics alone (**Bandyopadhyay and Mandal, 2014**).

Although, the market contains prebiotic supplements that claim the preference of administrating prebiotics combination over administrating one type only and few studies showed that the health benefits were boosted when prebiotics were used in combination (**Lecerf *et al.*, 2012**), no scientific literature has studied the benefit of combining the prebiotics together on the microbiota proliferation and the potential synergism that could developed by such combination.

Formerly, we have produced chitooligosacchride (COS) from the enzymatic hydrolysis of chitosan and its prebiotic stimulatory activity was confirmed (**Ismail *et al.*, 2020**). Generally, COS are either homo or hetero linear oligomers

of glucosamine and/or N-acetyl-D-glucosamine linked by β-1,4-glycosidic linkages with a degree of polymerization ranged from 2 to 20 units and with an average molecular weight less than 3.9KDa (**Muzzarelli., 1993**).
In the present study, we have evaluated for the first time to our knowledge the possible synergistic effect of combining COS with fructooligosaccharide (FOS), lactulose, and inulin on the growth of probiotics by calculating the combination index of each mixture under the study.

MATERIAL AND METHODS

Bacterial strains

Bifidobacterium lactis BB12 was obtained from Northern Regional Research Laboratory (NRRL), Agriculture Research Service, National Center for Agriculture, Peoria, Illinois, USA. *Lactobacillus helveticus* CNRZ 32 was supplemented from Centre National de RechercheZoo technique, Jouy-en-Josas, France.

Production of chitooligosaccharide

Chitooligosaccharide was produced by the hydrolysis of chitosan employing chitosanase enzyme produced from fermentation of shrimp byproducts using *Bacillus cereus* strain SSW1 as previously described by **Ismail., 2019.**

Assessment of possible synergism between chitooligosaccharide and commercial prebiotics

Two probiotic strains, *Bifidobacterium lactis* BB12 and *Lactobacillus helveticus* were grew on De Man, Rogosa and Sharpe broth (MRS) for 48h at 37ºC. Samples were taken and cell counts were determined by the pour plate method using MRS agar according to **Azmi *et al.*, 2012**.

Table 1 Prebiotic mixtures formulated along the study.

Combination number	Prebiotic 1		Prebiotic 2	
	Type	Concentration (w/v%)	Type	Concentration (w/v%)
C1	-	-	FOS	0.25
C2	-	-	FOS	0.5
C3	-	-	FOS	1
C4	COS	0.25	-	-
C5	COS	0.25	FOS	0.25
C6	COS	0.25	FOS	0.5
C7	COS	0.25	FOS	1
C8	COS	0.5	-	-
C9	COS	0.5	FOS	0.25
C10	COS	0.5	FOS	0.5
C11	COS	0.5	FOS	1
C12	COS	1	-	-
C13	COS	1	FOS	0.25
C14	COS	1	FOS	0.5
C15	COS	1	FOS	1
C17	-	-	Lactulose	0.25
C18	-	-	Lactulose	0.5
C19	-	-	Lactulose	1
C20	COS	0.25	Lactulose	0.25
C21	COS	0.25	Lactulose	0.5
C22	COS	0.25	Lactulose	1
C23	COS	0.5	Lactulose	0.25
C24	COS	0.5	Lactulose	0.5
C25	COS	0.5	Lactulose	1
C26	COS	1	Lactulose	0.25
C27	COS	1	Lactulose	0.5
C28	COS	1	Lactulose	1
C29	-	-	Inulin	0.25
C30	-	-	Inulin	0.5
C31	-	-	Inulin	1
C32	COS	0.25	Inulin	0.25
C33	COS	0.25	Inulin	0.5
C34	COS	0.25	Inulin	1
C35	COS	0.5	Inulin	0.25
C36	COS	0.5	Inulin	0.5
C37	COS	0.5	Inulin	1
C38	COS	1	Inulin	0.25
C39	COS	1	Inulin	0.5
C40	COS	1	Inulin	1

Countable number of the two probiotic strains were added separately to MRS broth and variable concentrations of COS, inulin (Sigma–Aldrich, USA), lactulose (EIPICO, Egypt) and fructooligosaccharide (Sigma–Aldrich, USA) were added either alone or in combination as shown in (Table 1). After incubation for 48h at 37ºC, samples were taken and the count number for each strain was examined by the pour plate method. Negative control was done by allowing the two strains to grow without any prebiotics for 48h at 37ºC.

Statistical analysis

The combination index (CI) was calculated based on Chou-Talalay method (**Chou., 2010**). Chou-Talalay method is mainly used to assess the synergism between pharmaceutical drugs, and the method was modified to evaluate the synergism between prebiotics. Compusyn software (ComboSyn. inc., Paramus, NJ) was employed to determine the CI using the following equations:
Median effect equation
$(F_a/F_u) + \{(C)/(C_m)\}^m$ (1)
Where Fa/Fu, C, Cm, and m are fraction affected/fraction unaffected, prebiotic concentration, median-effect concentration, and kinetic order, respectively.
Combination index equation
$CI = (P_A/P_{X,A}) + (P_B/P_{X,B})$ (2)
Where $P_{A/B}$ and $Px,_{A/B}$ represent the concentration of prebiotic used in combination to achieve x% effectiveness and concentrations of single prebiotic to achieve x% prebiotic effect, respectively.
The combined effect was considered additive if (CI = 1), synergism if (CI < 1), and antagonism if (CI > 1).
The statistical significance was analyzed using one-way analysis of variance. Paired t-test was used to evaluate the difference between two groups of data using a statistical software package (Statistical Analysis System, SAS Institute Inc., Cary, NC). Differences were considered statistically significant between related parameters for *P*-value equal to or less than 0.05.

RESULTS

Effect of combining chitooligosaccharide and fructooligosaccharide on the viability of *Lactobacillus helveticus*

The effect of combining COS and FOS on the *Lactobacillus helveticus* viability was evaluated (Figure 1A). The data showed that the *Lactobacillus helveticus* count was $3.55x10^7 \pm 2.6x10^6$ CFU/mL following the incubation for 48h without the addition of COS or FOS. When COS or FOS added without combination, the count slightly enhanced to reach $9.9x10^7 \pm 1.73x10^6$ CFU/mL and $1.7x10^8 \pm 1.73x10^7$ CFU/mL at concentration of 1%, respectively. A combination of COS and FOS at 1% for each, caused significant enhancement in the count to $1.26x10^9 \pm 1.15x10^7$ CFU/mL.

Effect of combining chitooligosaccharide and lactulose on the viability of *Lactobacillus helveticus*

It can be indicated from figure 1B that lactulose alone failed to enhance the viable count of *Lactobacillus helveticus* at concentration of 0.25%. At higher concentration, the viable count enhanced from $6.75x10^7 \pm 1.44x10^6$ CFU/mL to reach $1.17x10^8 \pm 1.73x10^6$ CFU/mL at a concentration of 1%. By adding COS to lactulose the viable count was gradually enhanced by increasing the concentration of both prebiotics to reach $2.37x10^8 \pm 3.75x10^6$ CFU/mL at a combination of 1% COS and 1% lactulose.

Effect of combining chitooligosaccharide and inulin on the viability of *Lactobacillus helveticus*

The *Lactobacillus helveticus* count after 48h incubation at 37ºC was $8.6x10^7 \pm 2.31x10^6$ CFU/mL (Figure 1C). Adding inulin alone slightly enhanced the viable count to reach $1.16x10^8 \pm 5.7710^5$ CFU/mL at 1% concentration. Similarly, adding COS alone slightly enhanced the viable count to reach $9.43x10^7 \pm 2.89x10^5$ CFU/mL at 1% concentration. Mixing COS and inulin in different concentrations steadily improved the viable count to reach $1.21x10^8 \pm 5.77x10^5$ CFU/mL at concentration of 1%.

Effect of combining chitooligosaccharide and fructooligosaccharide on the viability of *Bifidobacterium lactis*

The viable count of *Bifidobacterium lactis* after incubation for 48h at 37ºC either alone or in the presence of COS and/or FOS is presented in figure 2A. The data showed that in absence of COS or FOS, the viable count of *Bifidobacterium lactis* was $3.05x10^8 \pm 1.4x10^7$ CFU/mL following incubation for 48 h. In the presence of FOS or COS alone, the viability count was enhanced gradually depending on the FOS or COS concentration to reach $9.6x10^8 \pm 2.3x10^7$ CFU/mL and $9.6x10^8 \pm 2.4x10^7$CFU/mL for FOS and COS, respectively. When FOS was added to COS, the viable count was further enhanced with different magnitude

depending on the COS and FOS concentration. The maximum viable count was $1.06x10^{10} \pm 2.8x10^{8}$ CFU/mL at 1% concentration for both COS and FOS.

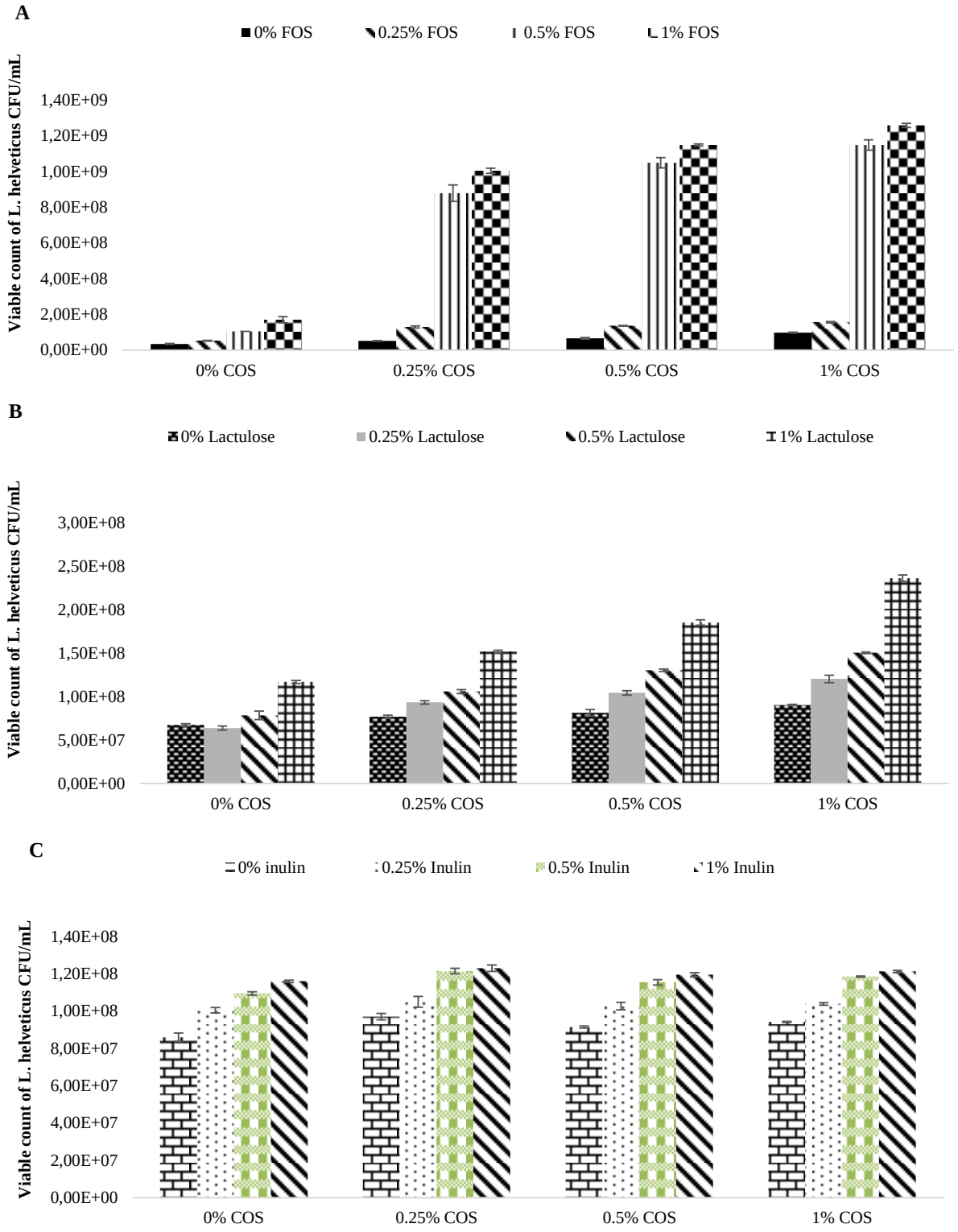

Figure 1 *Lactobacillus helveticus* viable count in the presence of (A) COS and/or FOS at different concentration (0.25%, 0.5%, 1%) (B) COS and/or lactulose at different concentration (0.25%, 0.5%, 1%) (C) COS and/or inulin at different concentration (0.25%, 0.5%, 1%). The effects of prebiotics were tested compared to prebiotic free media as negative control. The data display the mean ± standard error (SEM) of three replicas

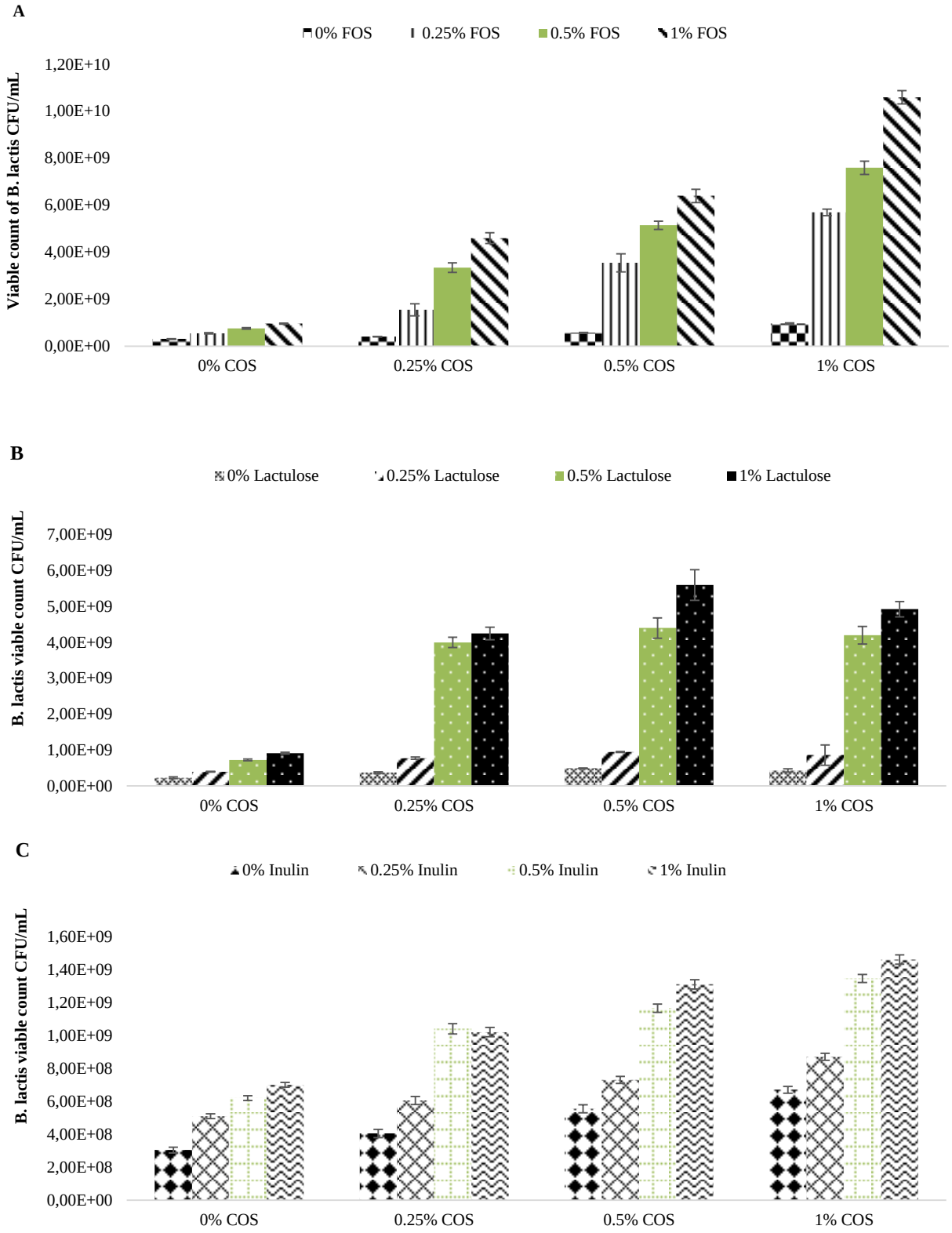

Figure 2 *Bifidobacterium lactis* viable count in the presence of (A) COS and/or FOS at different concentration (0.25%, 0.5%, 1%) (B) COS and/or lactulose at different concentration (0.25%, 0.5%, 1%) (C) COS and/or inulin at different concentration (0.25%, 0.5%, 1%). The effects of prebiotics were tested compared to prebiotic free media as negative control. The data display the mean ± standard error (SEM) of three replicas.

Effect of combining chitooligosaccharide and lactulose on the viability of *Bifidobacterium lactis*

It was observed according to Figure 2B, that the viable count of *Bifidobacterium lactis* in the absence of COS and lactulose was $2.3x10^8 \pm 2.12x10^7$CFU/mL after incubation for 48h at 37ºC. The viable count was slightly enhanced to $9.15x10^8 \pm 2.47x10^7$ CFU/mL and $4.3x10^8 \pm 4.6x10^7$ CFU/mL by adding either lactulose or COS, respectively at 1% concentration. By mixing both prebiotics together the viable count significantly increased to reach $4.93x10^9 \pm 2.12x10^8$ CFU/mL at a concentration of 1% for both COS and lactulose.

Effect of combining chitooligosaccharide and inulin on the viability of *Bifidobacterium lactis*

Chitooligosaccharide and inulin were combined at different concentrations ranging from 0.25% to 1% to study the potential effect on the viability of *Bifidobacterium lactis*. The data showed that the viable count was $3.05x10^8 \pm 1.77x10^7$ CFU/mL when the COS and inulin were not added to the culture media after 48h incubation period. The viable count was enhancement to $6.7x10^8 \pm 2.12x10^7$CFU/mL and $7x10^8 \pm 1.41x10^7$ CFU/mL when the COS and inulin were added separately at 1% concentration for each. However, significant improvement in *Bifidobacterium lactis* viability was observed when the two

prebiotics were combined together at concentration 1% for each of them to reach $1.46x10^9 \pm 2.83x10^7$ CFU/mL (Figure 2C).

Synergistic stimulatory effect of combining COS with other prebiotics on the viability of *Lactobacillus helveticus*

The synergistic effect of combining COS with any of FOS, lactulose, and inulin was evaluated by calculating the CI index of each combination at different concentrations of each prebiotic. Table 2, showed the CI index of all prebiotic combinations that were employed using *Lactobacillus helveticus*. Some combinations (C5, C6, C7, C10, C11, C13, C15, C24, C25, C27, C28, C37, C39 and C40) showed significant synergism, since the CI index was less than 0.5, while the rest showed moderate synergism with CI value between 0.5 and 0.9 except C35 which showed weak synergism, while C32 and C34 displayed weak antagonist effect.

Synergistic stimulatory effect of combining COS with other prebiotics on the viability of *Bifidobacterium lactis*

The synergism between COS and FOS, lactulose, and inulin on stimulating the viability of *Bifidobacterium lactis* was indicated in Table 3. The data reveled that most of the prebiotic combinations under study showed higher synergistic effect with CI value less than 0.5 except 5 combinations including C20, C23, C32, C35, and C38, which showed moderate synergistic effects, since their CI values were in the range of 0.5 to 0.9. The observed data indicated that no prebiotic combination under study showed weak synergistic effect, additive effect or antagonist effect.

Table 2 Combination index of COS combined with other prebiotics to stimulate *Lactobacillus helveticus* viability

Combination number	Combination index	Combination number	Combination index	Combination number	Combination index
C5	0.47117	C21	0.70047	C34	1.35724
C6	0.07636	C22	0.57795	C35	0.89053
C7	0.12863	C23	0.51641	C36	0.54018
C9	0.52143	C24	0.46669	C37	0.19331
C10	0.06314	C25	0.37991	C38	0.75520
C11	0.11431	C26	0.48507	C39	0.44894
C13	0.63763	C27	0.33880	C40	0.29365
C14	0.06091	C28	0.21790		
C15	0.10352	C32	1.34417		
C20	0.58669	C33	0.83611		

Table 3 Combination index of COS combined with other prebiotics to stimulate *Bifidobacterium lactis* viability

Combination number	Combination index	Combination number	Combination index	Combination number	Combination index
C5	0.20512	C21	0.04753	C34	0.29766
C6	0.04412	C22	0.07239	C35	0.62740
C7	0.02454	C23	0.64964	C36	0.17530
C9	0.05490	C24	0.04681	C37	0.18800
C10	0.02460	C25	0.04092	C38	0.62865
C11	0.01316	C26	0.06823	C39	0.18096
C13	0.03165	C27	0.02231	C40	0.16421
C14	0.01139	C28	0.00721		
C15	0.00039	C32	0.84925		
C20	0.62561	C33	0.17592		

DISCUSSION

The application of prebiotics as a nutrient supplement has been expanded over the last decade. Recent records provided by Global Prebiotic Association showed that the global sales of prebiotics supplements reached USD 5.5 billion in 2019 and is expected to reach USD 8.34 billion by 2026 (**Report and data., 2019**). Most of the prebiotic supplements in the market are in the form of prebiotic mixture that can selectively stimulate the gut microbiota. Despite the global market gross of prebiotics and the preference of most producers to fabricate the prebiotic supplements in mixture form, no data is available regarding the potential synergism that could be demonstrated by administrating more than one prebiotic. In our former work, we have produced COS that showed potent prebiotic activity compared to other commercial prebiotics such as inulin and FOS (**Ismail *et al*., 2020**). In order to further enhance the prebiotic activity of COS, in addition to expand its market value, we have mixed COS with different concentrations of commercial prebiotics followed by analyzing the synergism between the tested mixtures. The Chou-Talalay method is based on the median-effect equation derived from the mass action law theory. Since the median is a common link and universal reference point in biological systems, the Chou-Talalay method has considered the mechanism independent, drug unit independent and dynamic order independent (**Chou., 2010**). Consequently, the method is recommended to measure the synergism in any biological system as long as the biological effect and the bioactive compound concentration that can be measured numerically. To apply this method here, we have considered the viable count of the probiotic bacteria as the biological effect produced by the prebiotic concentrations under study. Besides using more than one type of prebiotic in the current research in different concentrations, we have also employed two different probiotic bacteria. Our objective was initially to examine whether the synergism between prebiotics is really existing and further to clarify if the synergism is related to the prebiotic type or is related to the probiotic strain. It was clear demonstrated that synergism is definitely exists between prebiotics as most of the combination under study showed CI below 1 with some combinations even reached CI closer to 0.1 that indicates extreme synergism. Comparing the CI of prebiotic combination within the same strain, the results obviously showed that prebiotic type is one of the factors responsible for the synergism between prebiotics. By exploring CI data on *Lactobacillus helveticus*, it was clear indicated that the combination of COS with FOS showed significantly lower CI (more synergism) than the combination between COS and lactulose at the same concentration level which in turn showed lower CI (more synergism) than the combination between COS and inulin. The same pattern was also observed with *Bifidobacterium lactis*. The identical pattern in the two strains indicated the significance of careful selection of prebiotic type when designing a prebiotic mixture for the market use. Another indication can be extracted from the data regarding the prebiotic concentrations, it was clear observed that most of the combinations that include 0.25% concentration from any prebiotic have shown lower synergism compared to the other concentrations (0.5% and 1%) suggesting that prebiotic concentration has an impact on the synergism between prebiotics. Finally, comparing the CI data between the two strains under study at the same prebiotic type and concentration level has definitely confirmed that the probiotic strain is another factor that determines the value of synergism between prebiotics. All the prebiotic combinations showed CI values on *Lactobacillus helveticus* higher than CI values on *Bifidobacterium lactis* except two combinations including C20, and C23. In our future work, we are planning to assess other types of prebiotics and evaluate a wide range of prebiotic concentrations to widen the knowledge regarding the synergism between prebiotics.

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