Pharmacokinetics of probiotic microorganisms in the gastro-intestinal tract

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Introduction

Gut health is currently getting much attention among researchers and public. This mainly because many of the physical health problems were found to closely connected to the improper functionality of gut. Over the years, study of the microbiological system in the gut has slowly uncovered the general effects brought to the host. The concept of probiotic appears when effort is made to modify or alter the composition of the microfloras in the gut to a remedial one by directly introducing the biologically important beneficial microorganism. Currently, the prebiotic strategy is proposed as an alternative mode to improve the well being of gut. This is the application of indigestible food ingredient that has impact on the metabolism of intestinal microorganisms. In the scientific literature, most of the data on prebiotic effect deals with inulin and fructooligosaccharides, and a range of commercial products have been available for many years. Due to the potential synergy between probiotic and prebiotic, foods containing a combination of these ingredients are often referred to as synbiotic. One may also expect the prebiotic to serve as a preliminary growth substance while introducing a special functionality of probiotic into the gut. This is especially important in view of the weakness of probiotic surviving through gastrointestinal tract to reach an allocated site such as small and large bowel. On the other hand, the correct pair of probiotic and prebiotic is designed to insure a successful establishment of probiotic in the gut among the present of the large microbial assemblage. This is true when the substrate affinity of the probiotic, for example *Bifidobacterium*, is varied according to species and even up to the strain level.

Materials and Methods

Peptone Yeast Glucose (PY-G) broth previously formulated by Holdeman and Moore (1977) for the cultivation of intestinal bacterial was modified for the screening of inulinase activity from the array of wild type *Bifidobacterium pseudocatenulatum*. The only changes were the carbon source (glucose) used to oligofructose as PY-O and inulin as PY-I with the addition of agar. Positive results were indicated by the yellow zone appeared on the agar due to bromecresol purple as the acid indication. The best strain was selected from this screening test and its maximum specific growth rate (μmax) was determined for the purpose of obtaining the suitable dilution rate in the continuous cultivation. The same μmax was found in the case of the challenge harmful microorganism employed (in this case: *E. coli* V157). In therapeutic study, two different concentrations of bifidobacteria were used to challenge the growth of *E. coli* V157 in the continuous system (10^5 cfu/mL and 10^8 cfu/mL). The higher concentration was prepared via consecutive propagation of bacterial cell in a batch system. The bacteria cells were harvested by refrigerated centrifugation (8000 rpm for 20 min at 2^0C). Pellet cell obtained was suspended in 5 ml peptone water (50 % glycerol, v/v) and was further kept in a 12 mL sterile syringe under refrigerated condition (-20^0C) before seeding for maximum 3 days. The continuous cultivation was initiated when the maximum number of *E. coli* V157 achieved, normally after 6 h of incubation. Bifidobacteria was inoculated into the system for at least after 3 resident times of *E. coli* V157 cultivation. The antagonistic activity of *B. pseudocatenulatum* F117 against *E. coli* V157 was further evaluated by growth profile, acidification activity, and organic acid production during interaction. For prophylactic study, the experimental conditions were the same except that *B. pseudocatenulatum* F117 was to be initially cultivated before seeding in *E. coli* V157. For both experiment, glucose (0.5 %) and oligofructose (0.5 % and 1.0 %) were the main parameter monitored to evaluate for synergistic effect of bifidobacteria and prebiotic on antagonism.

Results and Discussion

All *B. pseudocatenulatum* screened showed excellent growth on PY-O agar with high acid production. The yellow zone appeared to be large (≥25mm) and obvious. This observation was true to all wild type strains, *B. pseudocatenulatum* and also the commercial reference strain, *B. pseudocatenulatum* JCM 1200. *B. infantis* ATCC 15698 exhibited moderate growth on PY-O agar with small (15mm≤x<25mm) but clear yellow zone. *B. breve* ATCC 29720 and *B. longum* BB536 produced barely obvious zone (5mm≤x<15mm) on this agar which indicated poor growth. Meanwhile, all *B. pseudocatenulatum* demonstrated poor growth on PY-I agar but for strain F117 and JCM 1200, which displayed moderate growth. No yellow zone (<5mm) observed on PY-I agar for the rest of the three reference strains: *B. infantis, B. breve and B. longum*. This implicit in the absent of acidification activity by these strains. Therefore, *B. pseudocatenulatum* was suspected to possess high activity in utilizing oligofructose and inulin. In that respect, enzyme inulinase must be highly induced for the purpose of breaking down the specific β-(2-1) linkages in these molecules.

In batch culture, for *B. pseudocatenulatum* F117, μ_m was calculated as 0.22 h^{-1}, 0.25 h^{-1} and 0.19 h^{-1} in glucose, oligofructose and inulin respectively. However, for *E. coli* V157, μ_m was determined as 1.00 h^{-1}, 1.10 h^{-1} and 1.20 h^{-1} in the corresponding

medium. μ_m in range of 0.10 h^{-1} to 0.12 h^{-1} was employed in the continuous interaction of *B. pseudocatenulatum* F117 and *E. coli* V157.

In therapeutic study, pH constant at 6.80 was suspected to affect the growth of freshly seeded *B. pseudocatenulatum* F117, which unable to proliferate to a higher count. On the contrary, growth stimulation was observed when *B. pseudocatenulatum* F117 were introduced into this similar condition except further monitoring of pH. pH lowering was presumably due to increase in organic acids concentration when the population of *B. pseudocatenulatum* F117 have considerably elevated. Lactic acid, a central organic acid in associate with bifidobacteria for the typical bifid shunt though, was not measured. However, batch cultivation with the same growing conditions observed an intense amount of lactic acid produced particularly in the initial stage of growth. In summary, under-detection of this compound might be ascribed to poor growth which rendered in the lost of competition for substrates available compared to *E. coli* V157. In addition to that, lactic acid was detected to profusely induce in respond to cell growth for batch cultivation of *B. pseudocatenulatum* F117. Consequently, lactic acid might be of chemically important in terms of growth.

By substituting oligofructose as the principal substrate to glucose, two interesting observations were operated concurrently. A more rapid acidification activity in line with profound growth of *B. pseudocatenulatum* F117 was noted. An enhanced growth rate once *B. pseudocatenulatum* F117 was seeded into the continuous flow system was definitely a critical characteristic. It has further approved that oligofructose formerly as the best growth substrate to *B. pseudocatenulatum* F117 in batch cultivation performed equally well to accelerate growth in continuous cultivation. Again, this could be a considerable criterion of prebiotic to immediately serve as colonic food for particular probiotic and to the greater extends stimulate its growth.

The study with a lower *B. pseudocatenulatum* F117 dose (10^5 cfu/mL) in control exhibited a relatively analogous antagonism on *E. coli* V157 compared to a higher dose (10^x cfu/mL) used. Besides, the antagonism prevailed could be separated into two interesting phases. First, while *B. pseudocatenulatum* F117 was growing to the maximum count and next was after this maximum count achieved. It was found that appropriate *B. pseudocatenulatum* F117 numbers was rather important, as the antagonistic activity was constantly proved to be higher in the second phase of interaction for either bacterial dose employed. Nevertheless, both *B. pseudocatenulatum* F117 doses consumed equally same duration to achieve the maximum growth, thus seemingly implied that lower *B. pseudocatenulatum* F117 dose performed comparably excellent to a higher dose. In condition with oligofructose fortified medium, no observation with regard to shortening of span for *B. pseudocatenulatum* F117 to achieve the maximum growth. Nevertheless, a growth stimulation response was still being detected immediately after seeding of *B. pseudocatenulatum* F117 into this environment (the first 6h) compared to control with slower growth rate. Lactate production again was stimulated in this medium, as this compound has yet appeared in control at the same time even though a comparable count of *B. pseudocatenulatum* F117 was assessed in both conditions. Stimulation in lactate production was therefore seemed to have a significant utility both for the growth of *B. pseudocatenulatum* F117 and also the antagonistic effect.

Current study with higher oligofructose dose did not observe to improve the maximum growth of *B. pseudocatenulatum* F117 from a lower oligofructose dose assessed. Meanwhile, *B. pseudocatenulatum* F117 could hardly raise up to log 9 cfu/mL in count as was normally achieved in all other cases. However, the antagonistic effect received by *E. coli* V157 was somehow greatly induced. When the pH declination trend was determined with respect to the lower oligofructose dose used, no apparent justification could be done. In all, a higher organic acids concentration was much significant to address for such an effect.

In prophylactic study, experimental results demonstrated that oligofructose did not significantly increase the resistance of *B. pseudocatenulatum* F117 against colonization of incoming *E. coli* V157 when glucose was available as growth substrate. However, *E. coli* V157 was unable to sustain in these systems with *B. pseudocatenulatum* F117 observed to have higher stability in its viable counts. In PY-G medium, *E. coli* V157 has demonstrated sign of resistance after an earlier 12 h of growth reduction. This resistance lasted for 12 h before a more susceptible effect ensued. During this duration, acetic acid was found to have drastically decreased in concentration. This might infer that *E. coli* V157 was capable of metabolizing acetic acid as growth substrate. However, a further decrease in pH disabled this metabolism to continue. On the other hand, the rate by which *E. coli* V157 eliminated from the system was not enhanced because of prebiotic (PY-O medium), but a slow and constant rate prevailed. During this period (30 h), acetic acid was also decreasing in concentration, again deducing the metabolism of this compound by *E. coli* V157, especially when oligofructose was not a good energy source as discussed earlier on. Lowering of pH discouraged the growth of *E. coli* V157, and conversely this condition supported the growth of *B. pseudocatenulatum* F117. Nonetheless, the growth of *B. pseudocatenulatum* F117 was only limited to log 9 cfu/mL in this system.

Conclusions

Screening experiment on inulinase activity for oligofructose and inulin has obviously shown that although generally regarded as authentic prebiotic for *Bifidobacterium* spp., however, the fact could be only reliable to certain strains but not the overall species. Wild type *B. pseudocatenulatum* F117 was found the best inulin and oligofructose utilizer and was therefore selected for advance study. Batch cultivation revealed that *B. pseudocatenulatum* F117 was well grown in oligofructose fortified medium instead of glucose or inulin fortified medium. A much higher organic acids production mainly of lactic, acetic and formic acid further approved the unique characteristic of oligofructose and was thus chosen specifically as prebiotic in therapeutic and prophylactic study. In therapeutic study, basically, pH and organic acids such as acetic, lactic and formic acid were the essential inhibitory agent towards the growth of *E. coli* V157. Most relatively differ and higher was the production of lactic acid from the fermentation of oligofructose to that of glucose. This compound was found to be more important as an acidification agent and also the cytotoxic factor rather than the commonly known acetic acid. Also, at low pH condition, a higher organic acids concentration could render in a greater inhibitory effect. Nevertheless, these organic compounds were observed to profusely produce when the numbers of bifidobacteria was at its maximum. Therefore, the numbers of

bifidobacteria (cfu/mL) available for effective interaction was become rather important. In the study of bifidobacteria dose (cfu/mL) effect on the growth commencement, the observation was that low initial loads, 10^5 cfu/mL performed comparably effective to the higher dose, 10^8 cfu/mL. In both options, the significant antagonism was assessed when the maximum growth of bifidobacteria attained (10^9 cfu/mL). And, it happened that both bifidobacteria doses exhibited almost the same duration for the maximum growth achieved. Nonetheless, when oligofructose was used as the carbon source (rather than glucose), the antagonism displayed much earlier, even before the expected maximum growth due. In the prophylactic study, nevertheless, there wasn't obvious improvement of *B. pseudocatenulatum* F117 by oligofructose to resist the exogenously introduced *E. coli* V157 as compared to glucose as carbon or energy supply. The pH and organic acids production to this context were insufficient to address for such an occurrence. However, in therapeutic and prophylactic study, the antagonism was much enhanced when the oligofructose concentration used in PY-O medium increased (from 0.5 % to 1.0 %). A more dramatic antagonistic interaction was illustrated by the elevation of organic acids concentration along with rapid acidification. Nonetheless, too acidic condition was found detrimental to the growth of *B. pseudocatenulatum* F117.

Benefits from the study

The combination of probiotic (*B. pseudocatenulatum* F117 and prebiotic (oligofructose) that can fulfilled the criterion of synbiotic (synergistic pair) was found. The effect of oligofructose to stimulate the growth of bifidobacteria was further explained in terms of organic acid production. Suitable dose of bifidobacteria for effective therapeutic function could be employed. Oligofructose as prebiotic can work to improve the establishment of bifidobacteria community in the allocated niches of gut. All these studies effectively provide a guideline for future clinical study.

Patent(s), if applicable

B. pseudocatenulatum F117 and oligofructose as "synbiotic pair"

Stage of Commercialization, if applicable :

Nil

Project Publications in Refereed Journals

1. **Yazid A.M.**, Ali A.M., Shuhaimi M., Kalaivaani V., Rokiah M.Y. and Reezal A. 2000. Antimicrobial susceptibility of bifidobacteria. *Letters in Applied Microbiology* 31: 57-62.

2. **K.W.Yap**, Rezaei Sabet M, L.C. Lim, Shuhaimi M, Sipat A, Ali AM, Abdul Rahim M, Nur Atiqah AM, Yazid AM. 2003. Bile Salt Deconjugation by Bifidobacteria. *Bioscience and Microflora.* (In progress)

Project Publications in Conference Proceedings

1. **L.C.Lim**, Yazid AM, A, Ariff and M. Kharidah. 2002. Therapeutic effect of bifidobacteria against commensal *E. coli* with prebiotic effect using continuous flow culture system. In symposium C: Life Science. Malaysian Science and Technology Congress. Pg. 14, 12-14 December 2002, Hotel Kuching Hilton, Kuching, Sarawak.

Graduate Research

Name of Graduate	Research Topic	Field of Expertise	Degree Awarded *(e.g. M.SC/Ph.D.)*	Graduation Year *(or expected)*
Lim Long Chang	Synergistic effect of *Bifidobacterium* and Fructooligosaccharides on the antagonism against *Escherichia coli*	Food Microorganism	M.SC	2004
Maryam Rezaei	Cholesterol assimilation by *Bifidobacterium* spp in vitro and in vivo study	Food Biotechnology	PhD.	2001

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