ORIGINAL ARTICLE

Lowland and Highland Varieties of *Dioscorea esculenta* Tubers Stimulate Growth of *Lactobacillus* sp. over Enterotoxigenic *E. coli in vitro*

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ABSTRACT

Objectives. Probiotic supplementation often only leads to transient improvement in the gut microbiome. Potential prebiotics, such as the oligosaccharide-rich varieties of *Dioscorea esculenta* tubers, can potentially bridge the gap between supplementation and persistent colonization. Thus, this study aimed to assess the ability of *D. esculenta* tubers to promote the growth of probiotic *Lactobacillus* sp. *in vitro* selectively.

Methods. The prebiotic activity of the selected varieties of *Dioscorea esculenta* tubers was evaluated via competitive growth assay, wherein the ratios of probiotic *Lactobacillus* sp. over enterotoxigenic *Escherichia coli* (ETEC) or "prebiotic ratios" were compared following treatment.

Results. Negative control (0.9% NaCl solution) produced a ratio of 0.88, Lowland and Highland varieties produced ratios of 1.26 and 1.29, respectively, and positive control (inulin) produced 1.54. The two varieties had comparable ratios to one another ($p > 0.05$), and significantly higher ratios than the negative control ($p < 0.05$). Both varieties have significant prebiotic activity. Compared to inulin, the two varieties' prebiotic activity was 84% as effective.

Conclusion. Overall, the tubers promoted the growth of *Lactobacillus* sp. over ETEC. The crude tuber samples, given their availability and affordability, can be easily integrated into the local diet to contribute to the improvement of the general population's health.

Key Words: Dioscorea esculenta, Enterotoxigenic Escherichia coli, Inulin, Lactobacillus, Prebiotics

INTRODUCTION

Probiotics, broadly defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as live microorganisms that when administered in adequate amounts confer a health benefit on the host,[1] now constitute one of the most commonly consumed supplements globally. The increasing popularity of probiotic supplements largely emerges from a growing knowledge base about how probiotic microorganisms can exert beneficial effects. Current studies suggest that probiotics, such as *Lactobacillus* sp. and *Bifidobacterium* sp., may confer benefits to the host through their interactions not only with the host's gut microbiota but also with the host's tissues. *In vitro* and *in vivo* studies have demonstrated that probiotics can promote resistance to pathogen colonization by competing for nutrients and epithelial attachment sites,[2,3] by creating an acidic luminal environment,[4] by producing antimicrobial[5] and quorum quenching compounds,[6-8] by improving the intestinal

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mucosa's barrier function,[9] and by enhancing the host's innate and adaptive immune defenses.[10,11] In addition, it is claimed that probiotics possess the ability to produce systemic effects by producing bile acid hydrolases that promote cholesterol and triglyceride clearance,[12,13] by attenuating key stress and anxiety-related pathways,[14,15] and by modulating the host's overall immune response.[10,11] Although various studies have shown that the intake of probiotics has improved outcomes in patients with infectious diarrhea, inflammatory bowel disease, inflammatory bowel syndrome, and necrotizing enterocolitis, there are trials with comparable methodological quality that suggest the opposite, collectively generating an inconsistent overall conclusion about the clinical efficacy of probiotic supplementation.[16] Apart from limitations in methodology and data analysis, the conflicting results in literature may be attributed to individual differences in diet and baseline gut microbiome configuration, which are essential determinants of colonization. Several studies show that while supplementation leads to increased fecal probiotic concentration, it does not necessarily result in mucosal probiotic colonization.[17,18] Additionally, the shedding of probiotic microorganisms in stool significantly diminishes following cessation of supplementation in the majority of hosts.[19,20] One strategy that is hypothesized to bridge the gap between supplementation and persistent colonization is the consumption of prebiotics, defined by the ISAPP as substrates that are selectively utilized by host microorganisms conferring a health benefit.[21] Typically composed of oligosaccharides, prebiotics may be fermented by probiotics, and in the process, provide a selectively nutritive environment for both probiotic microorganisms and the healthy gut microbiota. It is postulated that a diet with a suitable prebiotic composition can increase the likelihood of persistent probiotic gut colonization, potentially eliminating even the need for continuous probiotic supplementation. Edible tubers, such as yams of genus *Dioscorea*, form oligosaccharide-rich storage organs that may easily be integrated into an individual's regular diet to achieve an optimum dietary prebiotic intake. It has been shown that inulin, an oligosaccharide with an established prebiotic activity, may be extracted from *Dioscorea esculenta* tubers sampled from East Java.[22] However, there have been no studies on the prebiotic activity of *D. esculenta* tubers when consumed as a whole. Furthermore, the prebiotic potential of the Philippine varieties of the *D. esculenta* remains scantily explored. *D. esculenta*, locally known as *tugi*, has two known locally cultivated varieties, Lowland and Highland, which produce tubers that differ in their morphological and chemical profiles. While both varieties of tubers are comparably rich in complex carbohydrates, the tubers of the Highland variety have been found to have higher total phenolic content.[23] This becomes relevant considering the most recent consensus statement of ISAPP on the definition and scope of prebiotics where plant phenols, along with polyunsaturated fatty acids, have been named candidate prebiotics because several health benefits have been associated with the metabolites produced by their biotransformation by the gut microbiota.[21] Yams are relatively easy to cultivate, and they remain to be a staple source of carbohydrates in rural areas across the country. The assessment of the prebiotic activity of *D. esculenta* tubers could provide a basis for the integration of edible tubers in the regular diet with the intent to provide a cost-effective strategy for the maintenance of healthy gut microbiome.

METHODS

Processing and *in vitro* digestion

The tubers were oven-dried and pulverized. Then for each variety, 5 grams of dry powder were dissolved in 90 mL of 0.9% saline solution. The generated solutions were subjected to *in vitro* digestion based on a protocol by Kho *et al.*[12] Initially, 6 mol/L HCl was added until a pH of 2.0 was reached, then the solutions were left to stand for 15 minutes. Following the addition of 0.01 g pepsin, the solutions were left to stand for 2 hours. Through the addition of 1 mol/L $NaHCO_3$, the pH was adjusted to 5.0. The solutions were left to stand for another 2 hours after the addition of 0.0025g pancreatin and 0.015g bile salts. Finally, the pH was adjusted to 7.2, using 0.5 mol/L NaOH. Inulin powder and normal saline solution (NSS) were processed similarly to act as positive control and negative control, respectively.

Bacterial strains

Lactobacillus sp., the probiotic bacteria for this study, was isolated from a commercial product via direct plating on *Lactobacillus* MRS Agar. At the same time, the enterotoxigenic *E. coli* (ETEC), the growth competitor, was obtained from the culture collection of the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. The isolated strains underwent biochemical testing and selective plating onto MacConkey agar and Eosin Methylene Blue agar for identity confirmation.

Determination of optimal dosage

Enterotoxigenic *E. coli* were inoculated in LB broth, and the processed yams were added in varying concentrations (1% - 5% w/v in increments of 1%) to determine the amount of sample to be used for the competitive growth assay. The set-ups were incubated at 37^{0}C for 48 hours, and enterotoxigenic *E. coli* colony forming units (CFUs) were quantified using the drop plate method on MacConkey agar plates. The ability of the yam samples to create a more favorable environment for the probiotic bacteria even when the conditions set were initially favorable for the pathogenic competitor provides a better measure of their prebiotic activity. Thus, concentrations that are expected to permit the optimal growth for the enterotoxigenic *E. coli* should be chosen.

Competitive growth assay

Lactobacillus and enterotoxigenic *E. coli* were inoculated into LB broth preparations containing *in vitro* digested samples. Inulin powder acted as the positive control, while NSS alone acted as negative control. Initial CFU ratios of the prebiotic *Lactobacillus* versus the enterotoxigenic *E. coli* were adjusted to 1:1, then prebiotic activity was ascertained by comparing the CFU ratios after treatment. Set-ups were incubated at 37^{0}C for 48 hours. *Lactobacillus* CFUs were quantified using the drop plate method on MRS agar plates, while MacConkey agar plates were used for enterotoxigenic *E. coli*.

Data analysis

Statistical analyses were performed on SPSS Statistics. A two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine statistically significant differences between and among treatments.

RESULTS

Optimal dosage for the growth of ETEC is at least 2% w/v

The logarithmic values of CFU/mL for 1% w/v sample concentration for both Lowland and Highland varieties were significantly lower ($p < 0.05$) than all other concentrations tested. There were no significant differences between and among 2% to 5% w/v sample concentrations, implying that the effect of the increasing concentration of sample added plateaued at 2% w/v. Due to the induction of a set bacterial log phase, growth may plateau regardless of the amount of excess substrate due to the accumulation of toxic byproducts that can hinder the initially rapid growth of the bacterial population. To provide a favorable condition for the pathogenic competitor, the concentration was chosen for the competitive growth assay was 2% w/v.

ETEC CFU/mL is highest in both *D. esculenta* tuber varieties

The logarithmic value of CFU/mL of enterotoxigenic *E. coli* was found to be highest in both varieties of *D. esculenta* tubers. There was no significant difference between the two varieties, but both significantly increased ($p < 0.05$) the logarithmic value of ETEC CFU/mL by 23% compared to the negative control (normal saline solution). Additionally, positive control (inulin) significantly reduced ($p < 0.05$) ETEC CFU/mL by 33% compared to the negative control. This reaffirms the prebiotic activity of inulin, which is the main ingredient in commercial prebiotics.

Lactobacillus sp. CFU/mL is highest in both *D. esculenta* tuber varieties

The colony count of *Lactobacillus* sp. was also found to be highest in both varieties of the *D. esculenta* tubers. There was no significant difference between the two tubers, and both significantly increased ($p < 0.05$) the logarithmic value of *Lactobacillus* sp. CFU/mL by more than 80% compared to the negative control. The positive control only increased the logarithmic value of *Lactobacillus* sp. CFU/mL by 18% versus the negative control ($p < 0.05$).

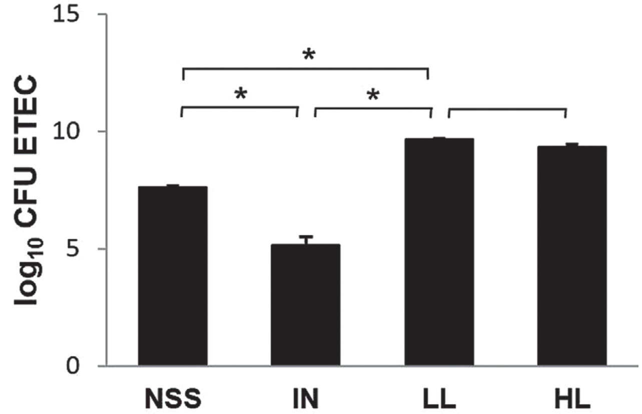

Figure 1. ETEC colony counts after 48 hours of incubation with different samples. This assay was performed in triplicate.

NSS - normal saline solution (0.9%); IN - 1% w/v inulin; LL - 2% w/v - Lowland variety; HL - 2% w/v - Highland variety.

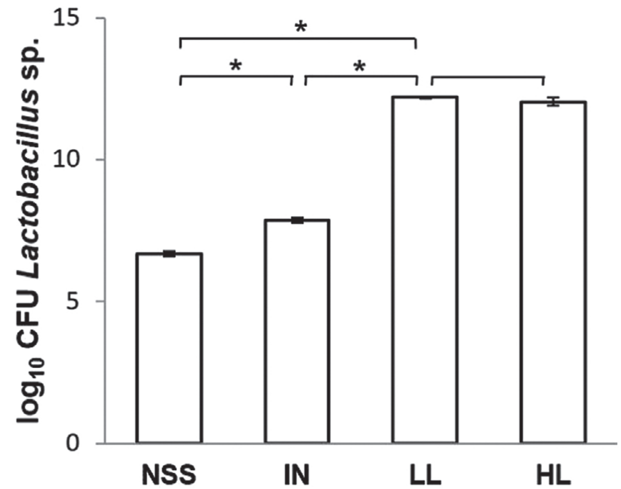

Figure 2. *Lactobacillus* sp.colony counts after 48 hours of incubation with different samples. This assay was performed in triplicate.

NSS - normal saline solution (0.9%); IN - 1% w/v inulin; LL - 2% w/v - Lowland variety; HL - 2% w/v - Highland variety.

Both Lowland and Highland tuber varieties showed prebiotic activity that is at least 84% as effective as inulin

Across all treatment conditions, the growth of *Lactobacillus* sp. and ETEC were significantly different from one another ($p < 0.05$). For the negative control, there was a higher number of ETEC colonies, and this was expected

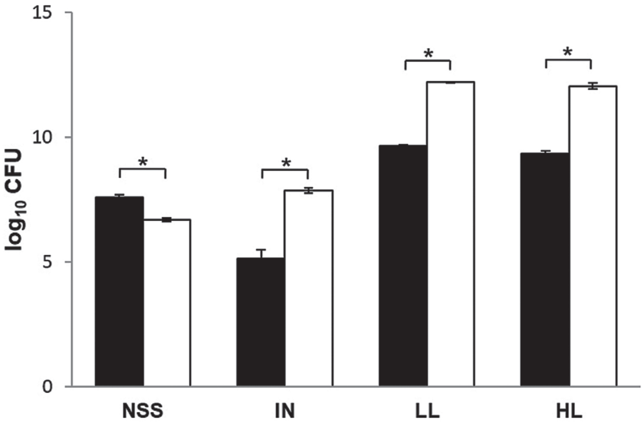

Figure 3. Comparison of *Lactobacillus* species (White) and ETEC (Black) colony counts after 48 hours of incubation with different samples. This assay was performed in triplicate.

NSS - normal saline solution (0.9%); IN - 1% w/v inulin; LL - 2% w/v - Lowland variety; HL - 2% w/v - Highland variety.

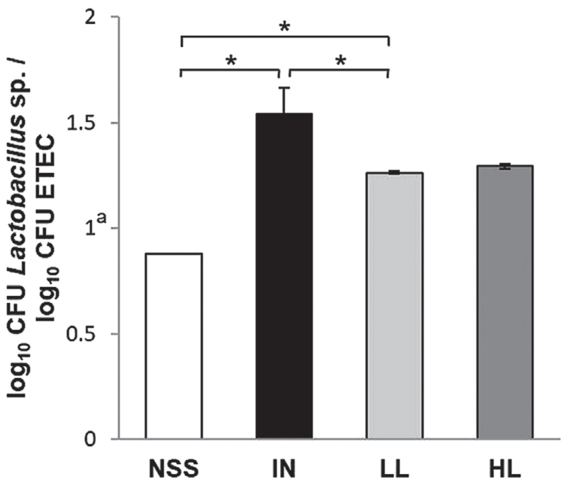

Figure 4. Growth ratios of *Lactobacillus* species over ETEC after 48 hours of incubation with different samples. This assay was performed in triplicate.

NSS - normal saline solution (0.9%); IN - 1% w/v inulin; LL - 2% w/v - Lowland variety; HL - 2% w/v - Highland variety. *[a] Starting ratio of Lactobacillus sp. and ETEC.*

as the primary conditions were optimized for the growth of ETEC. For the positive control, and both the Lowland and Highland variety treatments, there was a significantly larger amount of *Lactobacillus* sp. colonies ($p < 0.05$).

To evaluate the efficacy of each treatment condition in promoting the growth of the probiotic *Lactobacillus* sp. over enterotoxigenic *E. coli*, the ratios between the logarithmic values of bacterial CFU/mL per treatment condition were compared. Ratios were calculated as log *Lactobacillus* sp. CFU/mL/ log ETEC CFU/mL. A prebiotic ratio of 1 reflects an equal number of colonies for both types of bacteria, and a value greater than 1 is indicative of greater amounts of *Lactobacillus* sp. than ETEC, and thus, prebiotic activity. Negative control (0.9% NaCl solution) produced a ratio of 0.88, Lowland and Highland varieties produced ratios of 1.26 and 1.29, respectively, and positive control (inulin) produced a ratio of 1.54. The two varieties had comparable prebiotic ratios to one another ($p > 0.05$), and significantly greater prebiotic ratios than the negative control ($p < 0.05$). This indicates that both varieties of *D. esculenta* tubers have overall prebiotic activity. Although both variety treatments had larger absolute counts of *Lactobacillus* sp. colonies than the positive control, the ratios show that positive control still possesses the best prebiotic activity as both variety treatments had a significantly lower ($p < 0.05$) ratio than the positive control. When compared with the positive control, inulin, the two varieties' prebiotic activity was at least 84% as effective.

DISCUSSION

The effect of the *D. esculenta* tubers on the absolute colony counts of both ETEC and *Lactobacillus* sp. suggests that both varieties contain substances that are non-selectively nutritive. One such substance is starch, which may constitute up to 80% of the tubers' dry weight.[24] Starch extracted from *D. esculenta* tubers was shown to have a relatively smaller granule size, which is associated with the greater release of glucose with more of the surface area of the substrate exposed for the number of residual macronutrients enzymatic breakdown during digestion.[25] Nevertheless, the tubers were able to produce a significant overall prebiotic effect *in vitro* even when the treatment solutions have likely retained significant amounts of digested starch. This supports the idea that the selectively nutritive oligosaccharides, which are only available to *Lactobacillus* sp. for utilization, play a bigger role in the probiotic-competitor interaction under study. More importantly, it may be inferred that the inhibitory effect that probiotics and their metabolic byproducts can impose may negate the beneficial effects that can be provided by non-selectively nutritive substrates to pathogenic competitors, such as ETEC. As mentioned previously, the presence of an adequate supply of prebiotic substrates allows probiotics to proliferate and to subsequently inhibit the growth of pathogenic microorganisms through a diverse array of mechanisms. The ratio of selectively nutritive and non-selectively nutritive substances that is most optimal for the persistent colonization of probiotic microorganisms remains unknown, and further investigations are necessary to elucidate how the presence and the quantity of remaining macronutrients in the gut will affect the action of prebiotics upon supplementation. With this, the prebiotic activity of the tubers is hypothesized to improve *in vivo*. Given that the samples have only undergone *in vitro* digestion and not intestinal absorption, it is expected that, *in vivo*, a significant fraction if not most of the digested starch that the tubers contain will be absorbed by the host, leaving only the indigestible substances such as fermentable oligosaccharides for the gut microorganisms. The expected reduction in non-selectively nutritive substances will make it less likely for the ETEC population to catch up with the growth of *Lactobacillus* sp., thus, potentially increasing the overall prebiotic effect of the tubers. While the performance of the tubers may even improve *in vivo*, more research is needed to confirm this hypothesis and to factor in the multiplicity of determinants that influence the overall composition of the gut microbiota. The result of the competitive growth assay is limited to the interaction between ETEC and *Lactobacillus* sp. in a controlled environment, and the reproducibility of this result in an environment with other gut microorganisms has yet to be determined. The current opinion concerning balance in the host's gut microbiome is that it largely depends on the ratio of microorganisms colonizing the intestinal tract.[26] However, the role of absolute bacterial counts remains poorly understood. Currently, there are no clear-cut genera or species-specific limits as to what constitutes undergrowth or even overgrowth in the gastrointestinal tract, and the differential effect of substances that may produce the similar prebiotic ratios on the absolute count of both probiotics and pathogenic competitors needs further exploration. More importantly, further studies are needed to ultimately identify the impact of the tubers on both mucosal probiotic colonization and persistence post-supplementation.

Even if it has been shown previously that inulin constitutes only up to 20% of *D. esculenta* tubers' dry weight, both tuber varieties used in this study at 2% w/v have remarkably demonstrated a prebiotic activity that is at least 84% as effective as 1% w/v pure inulin. This implies that despite having an estimated inulin composition that is less than half of that of the positive control, the tubers have produced a comparable activity. This provides a potential lead to the role of other known and unknown substances found in the tubers in selectively promoting the growth of probiotic microorganisms. Previous studies have reported that *Dioscorea* tubers contain significant amounts of substances now considered as candidate prebiotics, such as phenolic compounds[23,27] and polyunsaturated fatty acids.[28] However, the extent of their contribution to the tubers' overall prebiotic activity remains unknown. Polyunsaturated fatty acids are thought to exert their prebiotic effect by providing a substrate to produce short-chain fatty acids, such as acetate and butyrate, which are essential mediators of several mechanisms by which probiotics provide beneficial effects, including the improvement of the host's intestinal epithelial barrier integrity and the activation of the host's immune response to invading infectious agents and even malignant cells.[29,30]

On the other hand, the majority of dietary polyphenols remain undigested after passing the small intestine and, therefore, provide substrates for probiotic microorganisms in the colon that may be converted into metabolites that may benefit the host.[31] While it has been shown that the tubers of the Highland variety have higher total phenolic content,[23] the two varieties had comparable prebiotic activity. Thus, it may be inferred that the phenols found in the tubers play a small role in promoting the growth of *Lactobacillus* sp. over ETEC. Aside from the well-known inulin, fructooligosaccharides, galactooligosaccharides, mannanoligosaccharides, and xylooligosaccharides may also be present in the tubers of *D. esculenta*, However, their contribution to the tubers' oligosaccharide composition needs to be confirmed. However, like phenolic compounds and polyunsaturated fatty acids, most of the previously mentioned oligosaccharides require more studies in human subjects to confirm their effects and to eventually fulfill their prebiotic status.[16]

This study has demonstrated the prebiotic activity of the tubers of both Highland and Lowland varieties of *D. escuenta in vitro*. While this necessitates confirmation in more complex *in vivo* models, the results allude to the possible utility of the tubers themselves as effective prebiotic supplements in place of isolating the specific known and candidate prebiotic compounds, such as oligosaccharides, phenols, and polyunsaturated fatty acids, before supplementation. This hypothesis provides a foundation for a more cost-effective and more convenient approach to the integration of prebiotic substances into an individual's diet. More importantly, it provides a basis for the investigation of simple dietary modification to promote and sustain persistent mucosal probiotic colonization.

CONCLUSION

Overall, the tubers promoted the growth of *Lactobacillus* sp. over ETEC, and this is confirmed by a comparison of the prebiotic ratios of the different treatments. While the positive control still produced the highest prebiotic ratio (1.54), the Lowland (1.26) and Highland (1.29) varieties were at least 84% as effective despite being administered as crude treatments. This allows for the utilization of the tubers' potential health benefits without the need for sophisticated purification methods. Easy access by the general population to the product due to their wide availability and relatively low price makes *D. esculenta* tubers a simple integration into the general diet. The prebiotic activity of *D. esculenta* tubers is mostly attributed to their relatively high inulin content. However, given the crudeness of the samples, there may be other substances that can explain their remarkable effects on the growth of probiotic species such as *Lactobacillus*. Thus, further characterization of the *D. esculenta* tubers should pave the way for a better understanding of not only how these tubers exert their prebiotic effect but also other potential health benefits of consuming these tubers.

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Statement of Authorship

Mr. Allan John R. Barcena and Ms. Aurora S. Nakpil – these two authors contributed equally to this research work. All authors have approved the final version submitted.

Author Disclosure

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