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Supplementing Yogurt with Probiotic Bifidobacteria to Counter Chronic Kidney Disease

Gerrit A. Stuivenberg [1,2], John A. Chmiel [1,2], Polycronis P. Akouris [1,2], Jessica White [3], Hannah Wilcox [1], Shannon Seney [1], Jeremy P. Burton [1,2,3] and Gregor Reid [1,2,3,*]

[1] Centre for Human Microbiome and Probiotic Research, Lawson Health Research Institute, London, ON N6A 4V2, Canada; gstuiven@uwo.ca (G.A.S.)
[2] Departments of Microbiology and Immunology and Surgery, Western University, London, ON N6A 3K7, Canada
[3] Department of Food and Nutritional Sciences, Brescia University College, London, ON N6G 1H2, Canada
* Correspondence: gregor@uwo.ca

Abstract: Chronic kidney disease (CKD) disproportionately affects populations in developing countries. In sub-Saharan Africa, CKD prevalence is high (12–23%) and is associated with cardiovascular manifestations. Uremic toxins, especially *p*-cresol and *p*-cresyl sulfate, are associated with the disease. Reducing uremic toxins in the body slows disease progression and improves patient outcomes. Probiotic *Bifidobacterium breve* HRVD521-US, *B. animalis* HRVD524-US, *B. longum* SD-BB536-JP, and *B. longum* SD-CECT7347-SP internalize *p*-cresol and improve longevity *in vivo*. In 2002, Tanzanian communities were taught to produce probiotic yogurt (Fiti®) supplemented with *Lacticaseibacillus rhamnosus* GR-1. This has expanded to over 100 community producers across the country. To produce yogurt that could reduce the burden of CKD by sequestering uremic toxins, we decided to test the addition of *p*-cresol-clearing bifidobacterial strains. By repeating the Fiti® production process performed in Tanzanian communities and adding a bifidobacterial strain, we found that they were successfully incorporated into the yogurt without any detrimental effect on sensory properties or viable counts. Three of the four strains significantly reduced *p*-cresol when added to a simulated colonic environment. In conclusion, this study has shown that Fiti® sachets provided to Tanzanian communities to produce yogurt can be supplemented with strains that can potentially confer additional health benefits.

Keywords: probiotics; yogurt; chronic kidney disease; uremic toxins; *p*-cresol

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1. Introduction

Chronic kidney disease (CKD) is a serious global health problem that affects more than ~10% of adults worldwide [1]. In African populations, the disease incidence is higher due to an increased prevalence of risk factors, including hypertension, genetic polymorphisms, and the sickle cell trait [2–7]. Due to these risk factors, the likelihood of developing CKD is almost doubled in sub-Saharan Africa, where upwards of 20% of the population is burdened by the condition [8–11].

The disease is characterized by the gradual loss of kidney function that impairs primary solute clearance and facilitates the accumulation of harmful compounds [12,13]. In the later stages of CKD, renal activity is completely inadequate in combatting the constant production of waste products, allowing them to persist in the body and thereby enhance the disease severity [14,15]. Certain renal replacement therapies, such as hemodialysis, exist to limit the accumulation of these harmful compounds [16], but their clinical efficacy often relies on early referral to a nephrologist at the onset of the disease [17]. However, due to a lack of resources and the high cost of these treatments, the majority of patients in sub-Saharan Africa are referred late, if at all [16,18–20]. This highlights the need for a

prophylactic strategy to address the CKD crisis in an economically sensitive fashion, with a particular focus on populations living in sub-Saharan Africa.

The build-up of gut microbiota-derived uremic toxins accelerates CKD progression [21–26]. *p*-Cresyl sulfate is an especially important toxin sourced from the bacterial metabolism of tyrosine and phenylalanine. Microbial fermentation of these amino acids in the gut yields the toxin precursor, *p*-cresol, which is absorbed from the intestinal environment to be sulfated in the liver, after which *p*-cresyl sulfate enters the circulation and binds albumin [27–33]. The accumulation of *p*-cresol and *p*-cresyl sulfate is known to induce oxidative stress and renal inflammation, decrease kidney cell viability, and enhance NADPH oxidase activity [34], each of which are manifestations commonly observed with the disease.

The gut microbiota of CKD patients is primed for *p*-cresol biosynthesis through the enrichment of toxin-producing bacteria, such as *Enterobacteriaceae* and *Clostridiaceae* [35,36]. The guts of these patients are also depleted of Lactobacillus and Bifidobacterium, two genera that are commonly used in probiotic formulations [35,37–39]. Therefore, the potential exists to administer strains from these genera to remedy microbial dysbiosis and uremic toxin accumulation. In studies testing this theory, the supplementation of probiotic bifidobacteria was consistently successful in slowing CKD progression and decreasing serum *p*-cresol [34,40–45]. The authors concluded that the findings correlated with a normalized intestinal microbiota [40,41]. Other explanations are feasible, such as the bifidobacteria directly sequestering *p*-cresol from the surrounding environment, as we have shown [27]. The data suggest that some strains of bifidobacteria are more effective than others at reducing gut microbiota-derived *p*-cresol, which points to the importance of proper strain selection in commercial probiotics.

In 2002, local women in Tanzania were taught to produce probiotic yogurt (Fiti®) supplemented with *Lacticaseibacillus rhamnosus* GR-1 [46–52]; this social enterprise has resulted in over 260,000 consumers having weekly access to Fiti® and Yoba-for-life, which utilizes a generic strain of *L. rhamnosus* GG. Considering ~20% of people in sub-Saharan Africa will develop CKD and probiotic yogurt has shown clinical efficacy against the disease [8–11,40], there is a unique opportunity to modify a fermented food for additional health benefits. Fiti® was chosen as the product of choice because of its availability to populations in sub-Saharan Africa, where it has been clinically proven to have probiotic properties. The present study was designed to test whether *p*-cresol-clearing bifidobacterial strains could be added to Fiti® yogurt without negatively affecting viable counts, taste, or texture. We hypothesized that the addition of *p*-cresol-clearing bifidobacteria would not impact the sensory properties of Fiti® and that the strains would maintain their toxin-clearing ability following yogurt production. A combination of in vitro techniques and sensory tests were used to assess the feasibility of this approach.

2. Materials and Methods

2.1. Chemicals

p-cresol was obtained from Thermo Fisher Scientific Canada (Mississauga, ON, Canada, #C040025G) and stored under appropriate conditions as defined by the manufacturer. Relevant concentrations of toxin were made in acetonitrile (ACN; Thermo Fisher Scientific Canada, Mississauga, ON, Canada #A996-4) on the day of experimental use.

2.2. Bacterial Culture Conditions

All bifidobacteria strains were received in monoculture from Seed Health (Los Angeles, CA, USA) and were verified by Gram strain. *Bifidobacterium breve* HRVD521-US, *Bifidobacterium animalis* HRVD524-US, *Bifidobacterium longum* SD-BB536-JP, and *Bifidobacterium longum* SD-CECT7347-SP were streak-plated from the frozen stock onto Bifidobacteria Specific Agar (BSA) [53] and incubated anaerobically (BD BBL™ GasPak™, BD Biosciences, Franklin Lakes, NJ, USA, #260678) at 37 °C under stationary conditions for 24 h.

2.3. Freeze Drying Bacteria

Single colonies were selected and inoculated in 50 mL of De Man, Rogosa, and Sharpe (MRS) broth with 0.5 g/L L-cysteine at 37 °C under anaerobic and stationary conditions for overnight growth. To produce experimental cultures for freeze-drying, the 50 mL overnight cultures were used to inoculate 750 mL of fresh MRS (with 0.5 g/L L-cysteine) that were then incubated anaerobically at 37 °C under stationary conditions for 24 h. A small amount of each bifidobacterial culture was removed and serially diluted. One hundred microliters of each dilution were then plated onto BSA plates in technical duplicate and incubated anaerobically at 37 °C under stationary conditions for 24 h. After 24 h, the number of visible colonies was counted to determine the colony-forming units (CFU) of the cultures before they were lyophilized. The 750 mL cultures of individual bifidobacteria were centrifuged at 6140× *g* at ambient temperature for 15 min to remove the supernatant and collect cells, which were then washed thrice with sterile 1 × phosphate-buffered saline (PBS). After the final centrifugation, the cells were suspended in 10 mL/L of cryoprotectant mixture consisting of 10% (*w*/*v*) skim milk and 10% (*w*/*v*) sucrose. The resuspended cells were allowed to rest for 30 min at room temperature prior to flash freezing at −80 °C. The frozen cells were then freeze-dried (Pharmaceutical Freeze Dryer, Harvest Right, North Salt Lake, UT, USA) with an initial freeze temperature of −31 °C and then at 12 °C until completely dry (~24–36 h). Lyophilized cultures were powdered, and recovery was determined via CFU.

To determine the CFU of the lyophilized bifidobacteria, 0.1 g of the powder was resuspended in 1 mL of sterile 1 × PBS. The resuspended cells were serially diluted, and 100 µL of each dilution was plated onto BSA plates in technical duplicate and incubated anaerobically at 37 °C under stationary conditions for 24 h. After 24 h, the number of visible colonies was counted for CFU calculations.

2.4. Production of Probiotic Yogurt Containing p-Cresol Reducing Bifidobacteria

The probiotic yogurt was produced using a slightly modified procedure from that previously described by Van Tienen et al. [50]. Briefly, whole milk (3.25% fat, Neilson, London, ON, Canada) was pasteurized at >80 °C for 30 min prior to being cooled to 37 °C. Once cooled, a single package of Fiti® starter culture (containing 1 g of freeze-dried probiotic *L. rhamnosus* GR-1 and *Streptococcus thermophilus* C106) was added. For the yogurt with bifidobacteria, an additional 1 g of either freeze-dried *B. breve* HRVD521-US, *B. animalis* HRVD524-US, *B. longum* SD-BB536-JP, or *B. longum* SD-CECT7347-SP was added. The samples were then incubated at 37 °C for 12 h. After the incubation, the yogurt was transferred to 4 °C overnight.

2.5. CFU Quantification of Yogurt Bacteria

To determine the CFU of the bacteria present in the yogurt, 1 g of fermented milk was homogenized into 10 mL of sterile 1×PBS. The homogenate was serially diluted, and 100 µL of each dilution was plated onto appropriate agar plates in technical duplicate. For *L. rhamnosus* GR-1, the homogenate was plated onto MRS agar and incubated at 37 °C for 24 h in 5% CO_2. For the bifidobacteria, BSA was used and incubated anaerobically at 37 °C under stationary conditions for 24 h. To quantify *S. thermophilus* C106, *S. thermophilus* isolation agar (STIA) was used [54,55] for plating, and the samples were incubated aerobically at 37 °C for 24 h. To ensure accurate results, pure cultures of all relevant bacteria were plated on MRS, BSA, and STIA and incubated under the abovementioned conditions. The pure cultures could only grow in the desired conditions, for example, bifidobacteria could only grow on BSA under anaerobic conditions and not on MRS in 5% CO_2 or STIA in an aerobic environment.

2.6. Sensory Evaluation

All yogurt samples were sensory evaluated for scent, taste, color, and texture by 12 panelists from the staff. The panelists had no prior experience in yogurt evaluation as to mimic an average consumer of the product. A hedonic scale from 1 to 10 was used. Scale 1

refers to extreme dislike, and scale 10 refers to extreme liking. Overall acceptability was calculated from the total score of the judged attributes. The yogurt samples were evaluated for pH as a proxy for acidity 24 h after production.

2.7. p-Cresol Clearance in a Simulated Colonic Environment

The ability of the yogurt supplemented with different probiotic bifidobacteria to reduce *p*-cresol in a simulated colon environment was assessed. Briefly, 1 g of milk fermented by the Fiti® culture and a bifidobacterial strain was homogenized into 10 mL of sterile 1 × PBS. The homogenate was subcultured (1:50) into a colonic medium to assess toxin clearance in an environment more like the human gut than traditional bacteriological media. The colonic medium was prepared as previously described [56,57]. Briefly, the culture medium was prepared in ddH_2O and consisted of (litre^{-1}): 5 g starch, 5 g peptone water, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 4 g mucin (porcine gastric type III), 3 g casein, 2 g pectin (citrus), 2 g xylan (oatspelt), 2 g arabinogalactan (larch wood), 1.5 g $NaHCO_3$, 1.25 g $MgSO_4 \cdot 7H_2O$, 1 g guar gum, 1 g inulin, 0.8 g cysteine, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts No. 3, 0.15 g $CaCl_2 \cdot 6H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.05 g hemin, 10 µL Vitamin K and 1 mL Tween 80. The pH of the medium was adjusted to 5.5 with 0.5 M NaOH and HCl solutions, as appropriate. Each tube was spiked with 0.2 mg/mL *p*-cresol. The concentration of *p*-cresol mimicked its physiological extremes found in the human intestinal tract [27,58]. The inoculums were incubated anaerobically for 24 h at 37 °C under stationary conditions.

2.8. Sample Preparation and HPLC Analysis

Samples from the simulated colonic environment were prepared for HPLC analysis using a previously described method [27]. Succinctly, each sample was centrifuged at 4500× *g* for 10 min at ambient temperature. The supernatant was collected and diluted 1:4 with HPLC grade water (Thermo Fisher Scientific Canada, Mississauga, ON, Canada, #W5-4). The diluted supernatant was mixed 1:2 with acetonitrile (Thermo Fisher Scientific Canada, Mississauga, ON, Canada, #A996-4) and allowed to rest for 10 min at 4 °C to precipitate proteins. The samples were then centrifuged at 13,000× *g* for 10 min at 4 °C, and the resulting supernatant was filtered (0.22 µm) into light-protected HPLC vials. Samples were stored at 4 °C for no more than 48 h prior to analysis. Standards were made fresh in pure acetonitrile and filtered (0.22 µm) into light-protected HPLC vials.

Every sample and standard were analyzed with an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) instrument equipped with a degasser (G1379A), quaternary pump (G1311A), autosampler (G1313A), and diode array detector (G1315B). All analyses were performed on an Agilent Poroshell 120 EC-C_{18} (4.6 by 150 mm inside diameter [i.d.]; 4 µm particle size) column at ambient temperature. The column was equipped with InfinityLab Poroshell 120 EC-C_{18}, 4.6 mm, 4 µm, HPLC guard. All acetonitrile and water used were HPLC grade. The mobile phase consisted of an isocratic mixture of acetonitrile and 50 mM/L ammonium formate buffer (pH = 3.3) (40:60 [*vol*/*vol*]) at a flow rate of 1 mL/min. The sample injection volume was 10 µL, and detection was performed at 222 nm. Run times were 7 min, with *p*-cresol eluting at ~4.2 min. Data were analyzed using ChemStation B.04.03. The peak area of samples was compared with the peak area of the external calibration curve (0.001 mg/mL to 0.5 mg/mL) to quantify *p*-cresol.

2.9. Statistical Analysis

All statistical comparisons were performed using GraphPad Prism 9.5.0 software. Data values were tested for normality using the Shapiro–Wilks test or the D'Agostino and Pearson normality test. Nonparametric data were statistically compared with an unpaired, one-way Kruskal–Wallis test, complemented with Dunn's multiple-comparison test. Normally distributed data were compared with an unpaired, one-way analysis of variance (ANOVA), complemented with Dunnett's multiple comparison test.

3. Results & Discussion

3.1. Viability of Lyophilized Probiotic Bifidobacteria

The percent viability of the freeze-dried bifidobacteria was determined by comparing CFU before and after lyophilization. The viability of the freeze-dried bifidobacteria was excellent, with each probiotic strain having a mean percent viability > 50% (Figure 1). The mean viability for each strain was as follows: *B. breve* HRVD521-US (65.36%), *B. animalis* HRVD524-US (57.74%), *B. longum* SD-BB536-JP (61.49%), and *B. longum* SD-CECT7347-SP (61.04%). This highlights the fact that toxin-clearing bifidobacteria can be freeze-dried efficiently with minimal loss of viable bacterial cells. Considering the Fiti® starter culture sachets consist of lyophilized bacteria; these findings suggest that these bifidobacterial strains can be delivered in the same manner and may be incorporated into the original product or added at the time of yogurt production.

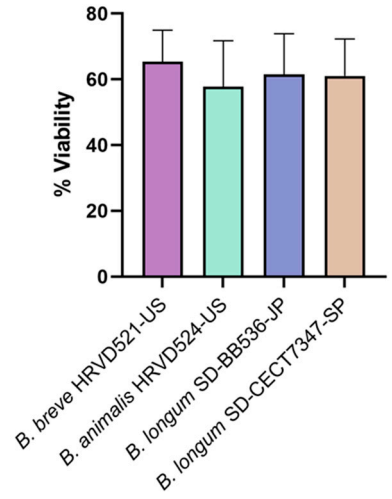

Figure 1. Percent viability of lyophilized bifidobacteria. The CFU of the cultures before freeze-drying was compared to the CFU of the lyophilized bacterial powder (n = 4).

3.2. Survival of Probiotic Bacteria in Yogurt Samples

To investigate the effect of probiotic bifidobacteria supplementation on the viability of the Fiti® strains, samples were assessed for total bacterial counts following production. The *L. rhamnosus* GR-1 and *S. thermophilus* C106 were recovered in sufficient quantities (CFU > 1×10^8) from every sample (Figure 2A). No bifidobacteria were found in the Fiti® controls, but their numbers reached more than 1×10^8 when added to the Fiti® (Figure 2A; one-way ANOVA; F = 18,633; $p < 0.0001$).

There was a slight, but statistically significant increase in *L. rhamnosus* GR-1 after adding the bifidobacteria (Figure 2A; one-way ANOVA; F = 13.53, $p < 0.0001$); yogurt samples supplemented with *B. animalis* HRVD524-US (p = 0.0016), *B. longum* SD-BB536-JP (p = 0.0004), or *B. longum* SD-CECT7347-SP ($p < 0.0001$) (Figure 2A; Dunnett's multiple comparison; α = 0.05) had more *L. rhamnosus* GR-1. A similar result was obtained for *S. thermophilus* C106 in yogurt samples supplemented with *B. breve* HRVD521-US (Figure 2A; one-way analysis of variance [ANOVA]; F = 4.169, $p < 0.0182$), which had significantly more *S. thermophilus* C106 (p = 0.0048) than Fiti® controls (Figure 2A; Dunnett's multiple comparison; α = 0.05). This suggested a slight stimulatory effect on the GR-1 and C106 strains with the addition of bifidobacteria. The reasons were not investigated here but are likely due to the lactobacilli and streptococci using metabolites from bifidobacterial growth. This corroborates a recent study where the oral supplementation of these strains increased the abundance of lactobacilli in the gut of *Drosophila melanogaster*, an in vivo model of *p*-cresol toxicity [27]. Bifidobacteria are known to enhance the growth of beneficial microbes through cross-feeding activities [59] by establishing networks that rely on the degradation of nutrients such as oligosaccharides, arabinogalactan, mucin, and

more [60–69]. This increases the abundance of short-chain fatty acid-producing bacteria, including lactobacilli [68]. Furthermore, the production of compounds such as lactic and acetic acid by bifidobacteria lowers the pH of the surrounding environment, as shown in Figure 2B, making it more conducive to the growth of lactobacilli [68,69]. Delivering short-chain fatty acid-producing bacteria to the human gastrointestinal tract also improves the availability of calcium and magnesium and inhibits pathogenic bacteria, including uremic toxin producers [62–65]. Thus, the oral administration of two short-chain fatty acid-producing bacteria, *Lactobacillus* and *Bifidobacterium*, could be significant for CKD patients [35,45]. Acetate produced by these two genera acts as an essential co-substrate for butyrate production by other microbes. Butyrate is the main energy source for colonocytes and has been shown to improve outcomes in colorectal cancer and chronic diseases [68]. Furthermore, a loss of butyrogenic bacteria is associated with CKD and enhanced toxin translocation from the intestinal environment [35]. It is therefore reasonable to postulate that the supplementation of acetate-producing probiotics could improve CKD outcomes by bolstering butyrate production, although this notion must be tested clinically for validation.

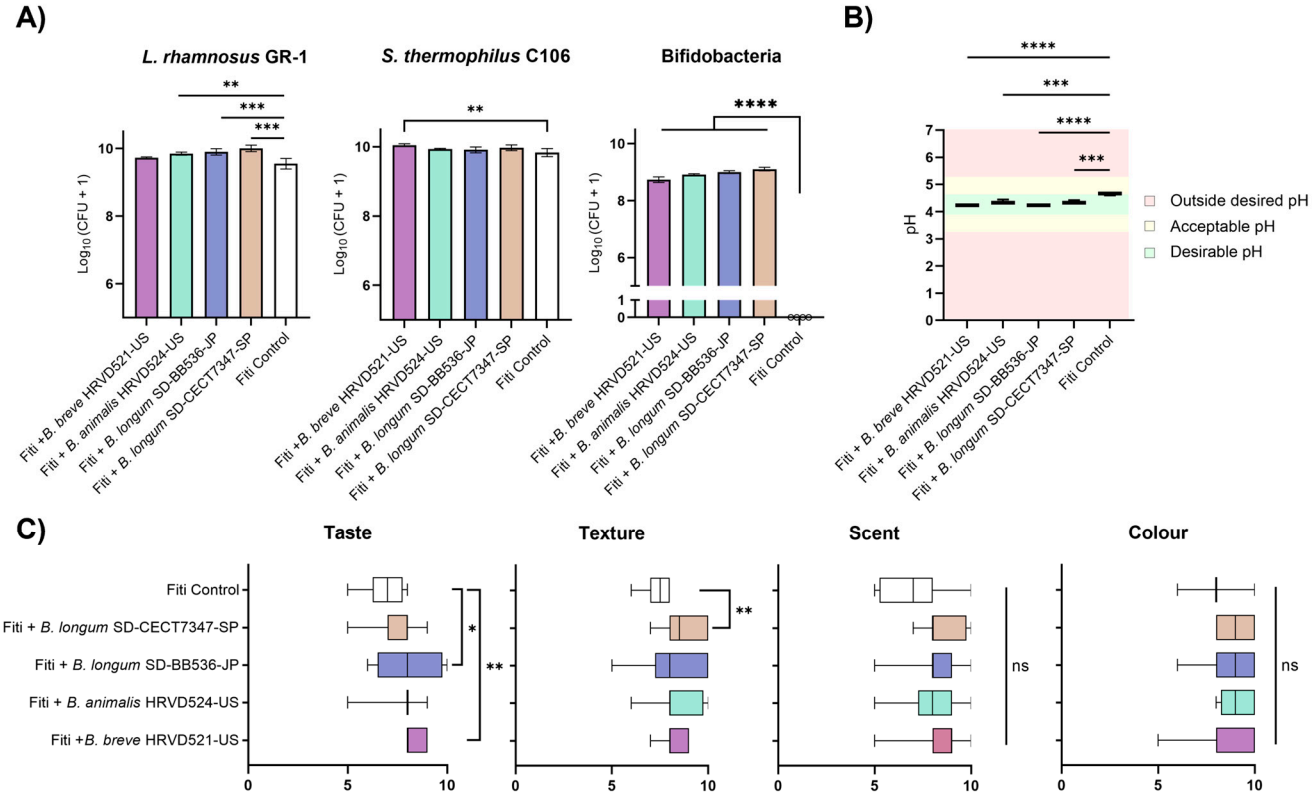

Figure 2. Supplementation of probiotic bifidobacteria improves the quality of Fiti yogurt. (**A**) CFU of *L. rhamnosus* GR-1, *S. thermophilus* C106, and *Bifidobacterium* spp. per yogurt sample plated on appropriate agar (n = 4). CFU data are displayed as the geometric mean of $\log_{10}$(CFU + 1) ± SD. (**B**) pH of yogurt samples 24 h after production (n = 4). (**C**) Sensory properties of the yogurt samples (n = 12). In the box plot diagrams (**B**,**C**), boxes represent the first and third quartile values, while black lines denote medians; whiskers encompass maximum and minimum values. All other data are displayed as the mean ± SD, unless otherwise stated. Statistical analyses shown are from a one-way ANOVA or Kruskal–Wallis test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [70]. As such, fortified yogurt retains the probiotic designation. The results show that Fiti® can be supplemented with other bacterial strains.

3.3. The pH of Yogurt Samples

A significant difference between the group means of pH (Figure 2B; one-way analysis of variance [ANOVA]; $F = 32.65$, $p < 0.0001$) was observed. In particular, the fermented samples containing *B. breve* HRVD521-US ($p < 0.0001$), *B. animalis* HRVD524-US ($p = 0.0002$), *B. longum* SD-BB536-JP ($p < 0.0001$), or *B. longum* SD-CECT7347-SP ($p = 0.001$) had a lower pH than the Fiti® controls (Figure 2B; Dunnett's multiple comparison; $\alpha = 0.05$). The decreased pH brought the bifidobacterial-containing samples into the "desirable" pH range as compared to the Fiti® controls, which resided in the 'acceptable' range [71,72].

All yogurt samples, including the Fiti® controls, had lower pH values due to lactose fermentation than the non-fermented milk used for production. The pH of pasteurized cow's milk is 6.5–7 [73–75], whereas all samples tested had a pH below 4.7 after fermentation (Figure 2B). Looking at the fermented samples specifically, the significant decrease in pH in the yogurt samples containing bifidobacteria compared to the Fiti® controls could be attributed to multiple factors. These include, but are not limited to, lactose fermentation into lactic acid and the *de novo* production of other short-chain fatty acids as mentioned above [68,76,77]. Granata et al. (1996) stated it is necessary to have yogurt at pH 4.0–4.4 to maintain flavor and texture [71]. This pH range occurred here, indicating the yogurts were of good quality. The mean pH of the Fiti® controls was 4.6. This is within the acceptable range, and the taste and texture are well received in Tanzanian communities. However, when the pH is higher, it can have detrimental effects on the flavor, texture, and survivability of lactobacilli [69,71,72,78].

3.4. Sensory Properties of Yogurt Samples

The sensory characteristics rated by the panelists are presented in Figure 2C. These include taste, texture, scent, and color. For taste, Fiti® controls had the lowest score when compared to samples supplemented with bifidobacteria. A significant difference between group medians was observed (Figure 2C; Kruskal–Wallis; $H = 15.47$, $p = 0.0038$). Specifically, the yogurt samples containing *B. breve* HRVD521-US ($p = 0.0013$) or *B. longum* SD-BB536-US ($p = 0.0144$) tasted better than Fiti® controls (Figure 2C; Dunn's multiple comparison; $\alpha = 0.05$). A similar observation was made for texture (Figure 2C; Kruskal–Wallis; $H = 11.20$, $p = 0.0244$) where the yogurt samples supplemented with *B. longum* SD-CECT7347-SP ($p = 0.0078$) received significantly higher scores than Fiti® alone. This may be attributed to the exopolysaccharides produced by bifidobacteria, which would increase the viscosity and enhance the body texture of the fermented milk [58,69,73]. In addition, the scent and color scores for the Fiti® controls were lower than all the yogurt samples containing probiotic bifidobacteria. Supplementation with B. *longum* SD-CECT7347-SP or *B. animalis* HRVD-524US resulted in the highest mean and median scores for scent and color properties, respectively, but the reason for this is unknown. These data highlight that probiotic bifidobacteria can easily be added to Fiti® and improve both the taste and texture of the product without negatively impacting other aspects of palatability for the consumer (i.e., scent and color).

3.5. Yogurt Supplemented with Probiotic Bifidobacteria Reduce p-Cresol in a Simulated Colon Environment

To identify whether yogurt supplemented with probiotic bifidobacteria could sequester uremic toxins from a colonic environment, yogurt samples were cultivated in the presence of *p*-cresol in a simulated colonic medium. Our analysis revealed a significant difference in *p*-cresol content between group means (Figure 3; one-way analysis of variance [ANOVA]; $F = 21.73$, $p < 0.0001$). There were lower amounts of *p*-cresol after 24 h of inoculation of yogurt with Fiti® strains plus one of the bifidobacterial strains in colonic medium compared to medium alone: *B. breve* HRVD521-US ($p = 0.0002$), *B. animalis* HRVD524-US ($p < 0.0001$) or *B. longum* SD-BB536-JP ($p = 0.0030$). The ability to reduce *p*-cresol was not observed in yogurt samples containing *B. longum* SD-CECT7347-SP ($p = 0.0886$) or the Fiti® controls ($p > 0.9999$). Similarly, the yogurt samples containing *B. breve* HRVD521-US ($p = 0.0002$),

B. animalis HRVD524-US ($p < 0.0001$) or *B. longum* SD-BB536-JP ($p = 0.0029$) had less *p*-cresol than the Fiti® controls (Figure 3; Dunnett's multiple comparison; $\alpha = 0.05$).

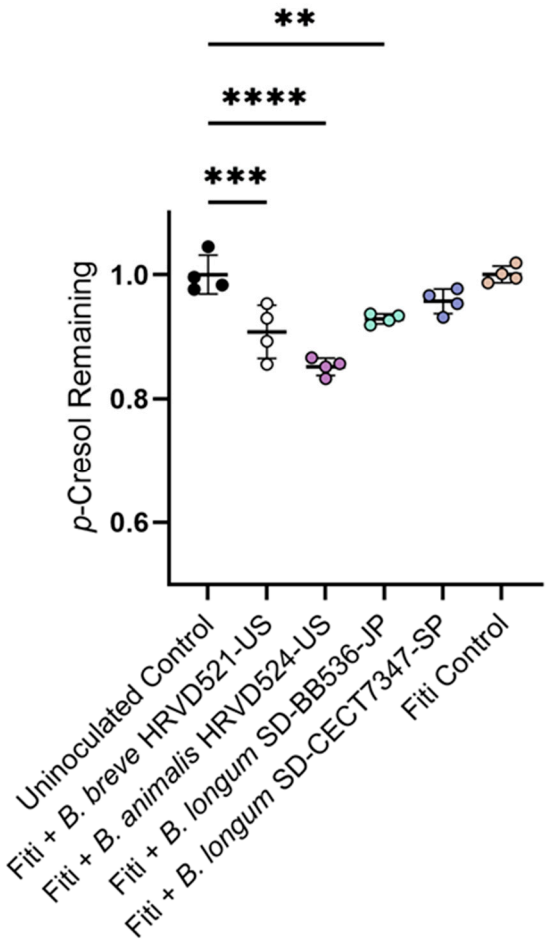

Figure 3. *p*-Cresol is depleted by probiotic bifidobacteria-containing yogurt within a mock colonic environment. Bacteria were cultured for 24 h in colonic medium spiked with 0.2 mg/mL *p*-cresol and the remaining toxin was quantified via HPLC ($n = 4$). Data are displayed as the mean ± standard deviation (SD). Significance was determined by one-way ANOVA; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

Reducing *p*-cresol availability in the human gut slows both CKD progression and the onset of comorbidities associated with the disease [21,22,79]. While this has led to remedies targeting the gastrointestinal tract, including inorganic phenol absorbents [80], there is growing evidence that probiotic therapies can leverage microbial diversity to reduce uremic toxins. Some strains of *Bifidobacterium longum* and *Bifidobacterium breve*, including one used in this study, have decreased serum *p*-cresol and slowed CKD progression in clinical studies [40,41]. However, the strain-specific contributions were muddled by the presence of prebiotics or additional probiotic strains that are known to promote the growth of other genera in the human gut. This highlights a need to assess these strains individually to better define their probiotic capabilities and clinical efficacy specific to CKD. While the bifidobacterial strains used here have been studied extensively to define their probiotic benefits ranging from immune education, gut-barrier integrity, pathogen inhibition, and micronutrient synthesis, their role in CKD has not been well defined [81–97].

While the potential of bifidobacterial supplementation for CKD patients has been documented, to our knowledge, no products are available with this claim or are being used in clinics. Indeed, this attribute is not listed on the Canadian and American websites of clinically documented probiotic strains and products (www.usprobioticguide.com, accessed on

17 March 2023; www.probioticchart.ca, accessed on 17 March 2023). Though it was initially thought that health benefits were attained by somehow balancing the intestinal microbiota, Stuivenberg et al. [27] challenged this notion by showing that the four bifidobacterial strains used in this study sequester *p*-cresol from their environment. We built on those findings by showing that *B. breve* HRVD521-US, *B. animalis* HRVD524-US, and *B. longum* SD-BB536-JP can clear *p*-cresol from a mock colonic environment [27,56,57]. The initial study investigating these four strains assessed toxin clearance in media with reduced nutritional content to promote the use of *p*-cresol as a carbon source [27]. In the present study, only three of the strains were able to reduce *p*-cresol from the nutrient-rich simulated colonic environment. This is not surprising, as *p*-cresol can be detrimental to both eukaryotic and prokaryotic cells [98–100]; therefore, it may only be used as a "last resort" nutrient source by capable microbes. Despite that fact, it is interesting to speculate how *p*-cresol could be a preferred carbon source for some strains of bifidobacteria, such as *B. breve* HRVD521-US, *B. animalis* HRVD524-US, and *B. longum* SD-BB536-JP, which still cleared it from the environment in the presence of excess nutrients and prebiotic sources. These findings highlight that not all probiotic strains, even from the same genus and species, have the same function.

Like the clinical investigations of probiotic bifidobacteria in CKD, the *p*-cresol clearing strains used here were delivered alongside another probiotic microbe, *L. rhamnosus* GR-1 [48,49,51]. Notably, the clearance of *p*-cresol relied on specific strains of bifidobacteria because neither the yogurt samples supplemented with *B. longum* SD-CECT7347-SP nor the Fiti® controls reduced *p*-cresol in the colonic mimic environment.

Fiti® and Yoba-for-life yogurts are currently accessible to over >260,000 people in sub-Saharan Africa on a weekly basis. Since these populations face the greatest risk for CKD development [8–11], the addition of *p*-cresol-clearing strains to Fiti® could have a dramatically positive impact on CKD incidence and severity in these regions at low cost and without needing to significantly modify the current production protocol. It would simply require one of the bifidobacteria tested here to be dried and made available in sachets to be added to the current strains. There is the potential to develop a probiotic yogurt that is accessible worldwide and can act prophylactically against CKD or as an adjunct in corroboration with other therapies. The next step is to perform a clinical trial to verify efficacy in a human cohort.

4. Conclusions

In conclusion, this study has shown that Fiti® starter-culture sachets being provided to African communities to produce yogurt can be easily supplemented with certain *p*-cresol-clearing *Bifidobacterium* strains for added health benefits. The addition of these strains improved the sensory properties of Fiti® and had a stimulatory effect on *L. rhamnosus* GR-1, a probiotic strain used in yogurt production. CKD patients have lower abundances of bifidobacteria and lactobacilli than healthy controls, and oral supplementation of these genera has shown promise in slowing disease progression [35,45]. Thus, delivering these genera via the consumption of Fiti® could have profound benefits for preventing and/or slowing the progression of CKD. Our analyses also revealed that three of the four bifidobacterial strains maintained *p*-cresol clearance following yogurt production and could clear the toxin from a simulated colonic environment. As such, this study highlights a new potential mechanism for reducing *p*-cresol in the colon and provides an inexpensive and effective way of delivering the strains in fermented food. As summarized in Figure 4, bifidobacteria have additional properties that are beneficial to CKD patients, including the inhibition of pathogen colonization [68,101], reducing toxin translocation [102], and enhancing beneficial commensals through the production of short-chain fatty acids [27,59,68]. While these data are promising, a clinical study is a crucial next step to verify the CKD-specific benefits of consuming bifidobacteria-supplemented Fiti® in humans.

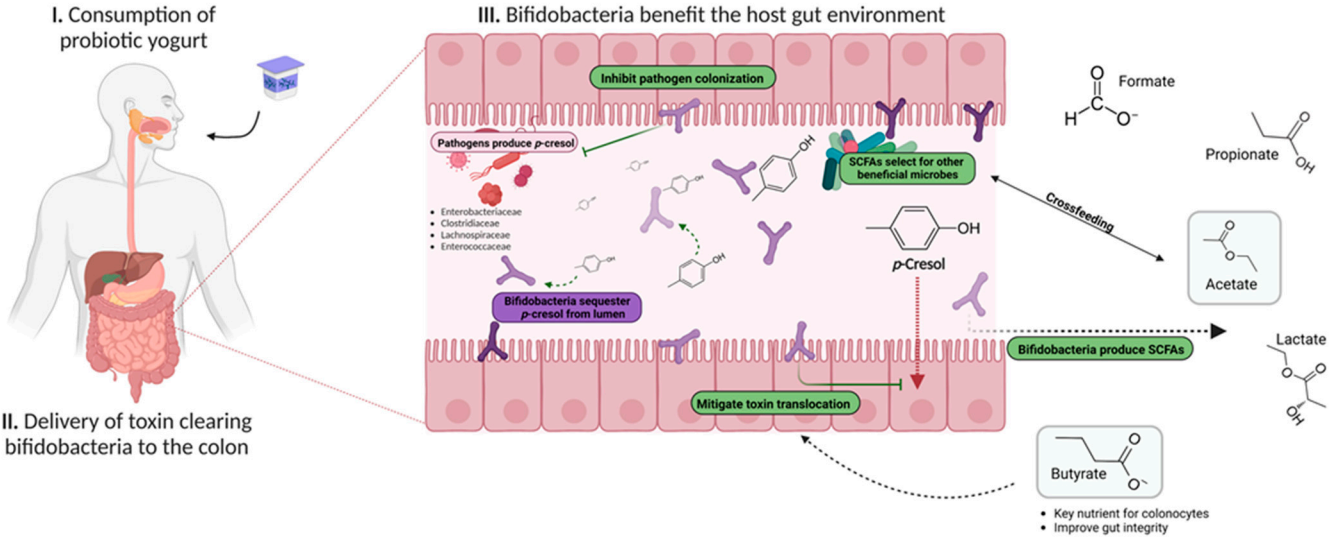

Figure 4. Summarizing the influence of bifidobacteria on promoting a healthy gut microbiota in CKD patients. The red box indicates the toxin-producing function of the dysbiotic gut microbiota in CKD patients, whereas green and purple boxes represent the known bifidobacterial mechanisms of action and the proposed function of *p*-cresol clearance, respectively.

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Conflicts of Interest: G.R. declares a conflict of interest, as he is a consultant to Seed Health. G.R. was not responsible for acquisition or analysis of the data, nor did G.R. prepare the original manuscript. G.A.S., J.A.C., P.P.A., J.W., H.W., S.S. and J.P.B. do not have any competing interests to declare.

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