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Artificial cell microcapsules containing live bacterial cells and activated charcoal for managing renal failure creatinine: preparation and *in-vitro* analysis

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Abstract

Activated charcoal was microencapsulated with *Lactobacillus acidophilus* 314 previously adapted for urea uptake. The creatinine removal capacity of this combination microcapsule was evaluated in-vitro in media simulating the small intestine. Results show that microcapsules containing both activated charcoal and *L. acidophilus* 314 demonstrated potential for decreasing creatinine. Interestingly, when co-encapsulating both activated charcoal and *L. acidophilus* 314 a smaller decrease in creatinine was observed than when encapsulating them separately. However, co-encapsulated microcapsules were more stable in various parts of the gastrointestinal system and survived longer in storage. These results suggest the feasibility of using microcapsules containing activated charcoal and probiotic bacteria as oral adjuvants for creatinine removal and provides a theoretical model for the use of these microcapsules to remove any unwanted metabolite.

Keywords: Artificial cell, activated charcoal, creatinine, microcapsules, oral therapy, renal failure, microbiome, live bacteria

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Introduction

Creatinine, a product constantly produced in muscles from the breakdown of creatine, is accumulated in the blood when renal excretory functions are impaired. This build up is indicative of late stage chronic renal failure and must be removed. Currently, dialysis offers an excellent treatment modality for chronic renal failure and end stage renal disease (ESRD) patients to lower creatinine and other unwanted metabolites. Dialysis, however, has substantial limitations. For example, it is associated with complications such as cardiomyopathy-related deaths (1) and peritonitis (2) particularly in children where the catheter has been in place for a long time (3). Although rare, surgical complications such as catheter site hemorrhages and intestinal perforation may also occur (4). As dialysis is a life-long treatment, it has been shown to also have negative psychological effects, inducing depression and anxiety in patients (5).

Despite constant advances in dialysis technology such as high-efficiency and high-flux dialysis, the rate of mortality and morbidity of kidney disease remains high (6). This has prompted researchers to shift away from improving dialysis methods to try other methods to treat renal failure, such as the oral administration of encapsulated bacterial cells (6, 7). Similarly, it was shown that oral administration of activated charcoal can be used to minimize epithelial tight junction damage and reduce oxidative stress and inflammation in chronic kidney disease animals (8). This article combines previous oral therapies and for the first time explores the use of co-encapsulated live bacterial cells and activated charcoal to remove renal failure metabolites.

Building on research done in the late 1990s to create semipermeable membranes capable of housing live bacterial cells (17), the use of probiotic microcapsules has grown mas-

sively. From reducing cholesterol levels (18), to disrupting the pathogenesis of hepatic encephalopathy (19), probiotics' effects on the gut microbiota cannot be underestimated. Encapsulating bacteria also allows for the mixing of different bacterial species creating novel probiotic blends. Different blends have been shown to target risk factors of metabolic syndrome (20) and Alzheimer's (21) *in-vivo* and in *drosophila melanogaster* respectively. All of this, in addition to previous research showing that probiotics reduce other uremic toxins, suggests the importance of probiotics both as an emerging field of research and as a potential therapy for renal failure.

Using oral microcapsules to remove uremic toxins is feasible due to the presence of these toxins in plasma, suggesting the exchange of uremic metabolites between the lumen of the GI tract and its capillaries (9). This exchange was well-characterized in the mid-1900s and prompted researchers to experiment on the feasibility of intestinal perfusion as a means of removing uremic toxins to manage renal failure (10, 11). However, as intestinal perfusion procedures need to be done at frequent intervals to prevent the build-up of uremic toxins such as creatinine, they are both expensive and lower the quality of life of patients. Another alternative is to orally administer encapsulated indigestible adsorbents for the removal of uremic toxins from the GI tract (12, 13). Such adsorbents include: activated charcoal as well as various carbon and starch nanoparticles (13, 14). Activated charcoal, in the concentrations used in this experiment, is a well-established nontoxic adsorbent for creatinine and uric acid (15, 16).

The aim of this study is to co-encapsulate both activated charcoal and metabolically induced *L. acidophilus* 314 cells and examine their efficacy of creatinine removal in in-vitro conditions simulating those of the small intestine. Ideally this co-encapsulation would provide a model for the removal of other unwanted renal failure metabolites.

Materials and Methods

Chemicals

Sodium alginate (low viscosity), creatinine and urea were purchased from Sigma-Aldrich, USA. Chitosan (low viscosity) was obtained from Wako Chemicals, Japan. Activated charcoal (Norit E Supra USP) was obtained from Norit Americas Inc., USA. Ox gall (dehydrated Fresh Bile) was obtained from Difco, USA, and pancreatin purchased from Acros Organics, USA. All other chemicals were of analytical reagent grade and not purified further before use.

Metabolic induction of *L. acidophilus*

Lactobacillus acidophilus 314 was obtained from ATCC. Bacterial cultures were maintained in Lactobacilli MRS Broth (Difco, USA) overnight at 37 ˚C in anaerobic conditions. *L. acidophilus* was cultivated in 1.5 ml MRS broth, with urea levels starting from 50 mg/Dl, and increasing in increments of 0.3 g/Dl to 2.7 g/Dl. The urea solution was filtered through 0.22 µm filter and added to autoclaved MRS broth. At each urea increment, the bacterium was cultivated for several passages for adaptation, before screening for healthy colonies on modified MRS agar plates enriched with a 0.3 g/dl higher urea concentration. The colonies were then re-inoculated into MRS broth at the higher urea concentration of the modified agar plate. This process was repeated until 2.7 g/Dl urea concentration (corresponding to 120 days), after which further increases in urea resulted in a bacterial growth too scant for practical use. The induced *L. acidophilus* was re-adapted to a lower urea level – 150 mg/Dl – similar to pathological levels in renal failure patients and used for *in vitro* experiments.

Microcapsule preparation

Chitosan-alginate microcapsules containing bacterial cells and/or activated charcoal were prepared based on microencapsulation procedures described in a previous report (22). *L. acidophilus* was grown in 150 mg/Dl urea-enriched MRS solution, harvested at late log phase, and collected by centrifugation at 10 000 *g* for 20 minutes at 4 ˚C. The media solution was discarded, and the cell mass washed three times with physiological solution (0.85 % (w/v) sodium chloride). 1.65 % (w/v) sodium alginate in physiological solution (PS) was sterile filtered through a 0.22 µm filter. 0.9 g *L. acidophilus* cells were re-suspended in alginate/physiological (PS) (9:1, v/v) reaching a final volume of 30.0 Ml. The bacterial suspension was extruded through a 600 µm diameter nozzle with pressurized nitrogen using an INOTECH encapsulator that was adjusted to a frequency of 450 Hz, a voltage of 0.25 Kv and an output pressure of 0.2 bar. Minute irregularities in the air stream resulted in the formation of two different sizes of microcapsules: 99 % egg shaped or oblate ellipsoidal microcapsules and 1 % larger, spherical microcapsules. The average diameters (minor diameters) of the 99 % microcapsule population are as follows: empty microcapsules: 619 µm ± 36 µm, *L. acidophilus* microcapsules: 628 µm ± 24 µm, activated charcoal microcapsules: 629 µm ± 28 µm and microcapsules containing both *L. acidophilus* and activated charcoal: 640 µm ± 23 µm, n=30. **Fig. 1** contains photomicrographs of the microcapsules under a light microscope. The droplets were stirred gently in chilled calcium chloride solution, 1.0 M (w/v), for 30 minutes and allowed to gel at 4 °C for 2 hours. Chitosan-alginate microcapsules were prepared by immersing the alginate droplets in a 0.5 % (w/v) chitosan solution in 0.5 % acetic acid (w/w) that is pH adjusted to 4.6, for 30 minutes. Microcapsules formed were washed and stored in PS at 4 ˚C until further use.

Combination microcapsules containing bacterial cells and activated charcoal were prepared according to the procedure described above, except that activated charcoal was added to the bacterial-alginate mixture prior to droplet extrusion. To test the reaction of the microcapsules in a simulated GI medium containing creatinine, the test groups include two negative controls: a) without microcapsules and b) empty microcapsules containing neither bacteria nor activated charcoal, and three test groups: c) *L. acidophilus* microcapsules, d) activated charcoal microcapsules and e) combination microcapsules containing both *L. acidophilus* and activated charcoal. Across the

Table 1. InoTech Encapsulator settings for the encapsulation of a) empty microcapsules, b) activated charcoal microcapsules, c) *Lactobacillus acidophilus* microcapsules, d) microcapsules containing both activated charcoal and *Lactobacillus acidophilus*.

Parameters	Microcapsules containing			
	Empty microcapsule	Microcapsule containing activated charcoal	*L. acidophilus* 314	*L. acidophilus* 314 & activated charcoal
Frequency (Hz)	450	420	430	420
Voltage (Kv)	0.25	0.197	0.25	0.197
Output pressure (bar)	0.3	0.25	0.2	0.25
Drop Height (inch)[a]	2.0	1.0	1.5	1.0

[a] The height refers to the drop height measured from the nozzle tip to the surface of the calcium chloride solution.

test groups containing the bacterial species, *L. acidophilus* was standardized in the microcapsules at 1.1 x 10^9 CFU/mL (colony forming units/mL) and 1.2 x 10^9 CFU/mL for the studies at pH 6.4 and pH 7.8 (to simulate conditions in the proximal and distal intestine respectively); while encapsulated activated charcoal was standardized at 54 mg for the studies at both pHs. Depending on the contents of the alginate mixture, the encapsulator settings differ for the extrusion of droplets and are summarized in **Table 1**.

In-vitro microcapsule studies in simulated gastric reaction media

Microcapsules were immersed in simulated GI media (consists of a carbohydrate-based diet with 1.0 g arabinogalactan/L, 2.0 g pectin/L, 1.0 g xylan/L, 3.0 g starch/L, 0.4 g glucose/L, 3.0 g yeast extract/L, 1.0 g peptone/L, 4.0 g mucin/L and 0.5 g cysteine/L. Pancreatic juice (containing 6.0 g ox gall/L, 0.9 g pancreatin/L and 12.0 g sodium bicarbonate/L) is added at 27.8 mL/100.0 mL simulated food mixture and is pH adjusted to simulate different sections of the small intestine. 12.5 mL creatinine is added per 100.0 mL simulated GI media. The microcapsules in each test group were immersed in 10.0 mL of the simulated GI media and incubated at 37 ˚C and anaerobic conditions for 48 hours. 1.0 mL of the synthetic media was sampled at 0, 10, 16, 24, 36 and 48 hours. The samples were centrifuged at 10 000 *g*, 4 ˚C for 10 minutes to obtain the supernatant for further analysis. Creatinine concentrations were determined using the 911 Hitachi Blood Chemistry Analyzer.

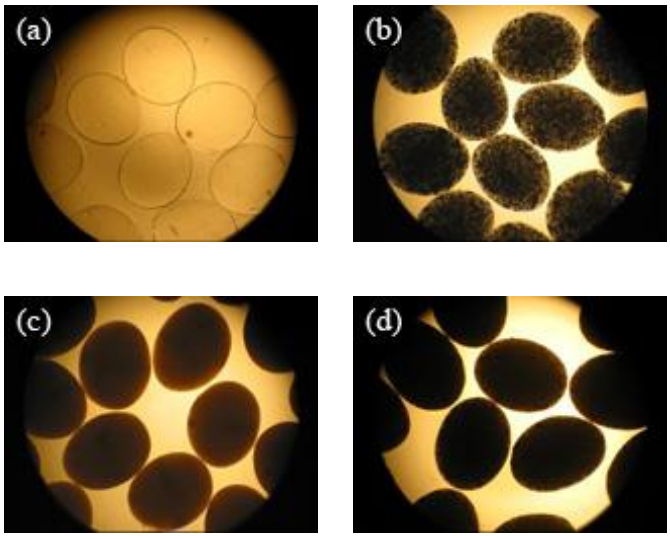

Figure 1. Photomicrographs, under a light microscope, of (a) "empty" microcapsules, microcapsules containing (b) activated charcoal, (c) *L. acidophilus* (d) activated charcoal and *L. acidophilus*. Magnification: 47X. Diameters across the microcapsule (a) 619 ± 36 µm, (b) 629 ± 28 µm, (c) 629 ± 24 µm, (d) 640 ± 20 µm.

Bacterial viability in storage conditions

Using the microcapsules immediately after its production as in this study is not always feasible, therefore it is important to test for bacterial viability in storage conditions, specifically in media at 4 ºC. This study involved two test groups: *L. acidophilus* microcapsules and the combination microcapsules containing *L. acidophilus* and activated charcoal. 4 g of microcapsules was immersed in 18 mL storage medium, which was either a) 10 % MRS medium enriched with 150 mg/dL urea 90 % PS solution or b) PS solution. 0.1 mL of microcapsules was sacrificed and plated at various dilutions to determine CFU counts in 1.0 mL at 0, 1, 2, 4, and 8 weeks.

Results and Discussion

The well-established adsorbent properties of activated charcoal and the inductive potential of bacteria have motivated their co-encapsulation for the removal of the uremic toxin creatinine in the GI tract. *In vitro* studies were focused on two pHs (pH 6.4 and 7.8) representative of the proximal and distal sections of the small intestine. Since exchange of molecules between blood capillaries and the GI cavity is the highest in the small intestine, the encapsulated bioreactors are likely to be most effective within this section at the pHs of 6.4 and 7.8. To visualize the efficacy of the microcapsules in decreasing creatinine, val-

Table 2. Calculated values[b] showing moles of creatinine removed per gram microcapsules

Microcapsule groups	Creatinine removed (per gram microcapsules)	
	Ph 6.4 (µmol/g)	Ph 7.8 (µmol/g)
Empty microcapsules	0.34	0.84
Activated charcoal microcapsules	1.26	1.78
L. acidophilus 314 microcapsules	0.71	0.83
L. acidophilus 314 & activated charcoal microcapsules	0.97	1.40

[b]The calculation is as follows: The number of moles of creatinine in the 10 Ml reaction media is first calculated by the equation: *Y* moles/1000 Ml x 10 Ml = *y* moles. The number of moles of creatinine at the end of the 48-hour experiment is also calculated (*x* moles). Their difference gives the total amount of creatinine reduced ((*y* – *x*) moles). When divided by the wet weight of microcapsules used, this yields the moles of creatinine removed per gram microcapsule.

ues are displayed in terms of the number of moles of creatinine removed per gram microcapsules were calculated and are shown in **Table 2**.

Effect of capsules on creatinine exposure at pH 6.4 (proximal section of small intestine)

After incubating the microcapsules in a simulated GI media of the proximal and distal sections of the small intestine for 48 hours in anaerobic conditions, it was shown that *L. acidophilus 314* microcapsules removed 204 µmol/L creatinine compared to creatinine removal of 103 µmol/L by empty microcapsules. This suggests that *L. acidophilus 314* may be able to metabolize creatinine, and that the empty capsules were able to trap creatinine following its diffusion into the alginate matrix, thus ensuring the complete removal of creatinine from the GI tract in fecal excretion after 24 hours. The largest removal of creatinine was achieved by activated charcoal microcapsules, where 379 µmol/L creatinine was removed in the same period.

From **Fig. 2**, the combination of *L. acidophilus* 314 and activated charcoal is not a superposition of their individual effects, where only 309 µmol/L ccreatinine was removed compared to 379 µmol/L by activated charcoal microcapsules. This may be due to physical obstruction of the pores on activated charcoal particles by *L. acidophilus* 314, resulting in the inability to utilize the full capacity of the adsorption area.

From **Table 2** shown earlier, the creatinine removal capacities of the microcapsules are compared per gram microcapsules, and these values support the discussion above. Activated charcoal microcapsules removed creatinine at 1.26 µmol/g, and *L. acidophilus* 314 microcapsules removed creatinine at 0.71 µmol/g. The combination microcapsules showed a creatinine removal at 0.97 µmol/g. This decreased creatinine removal ca-

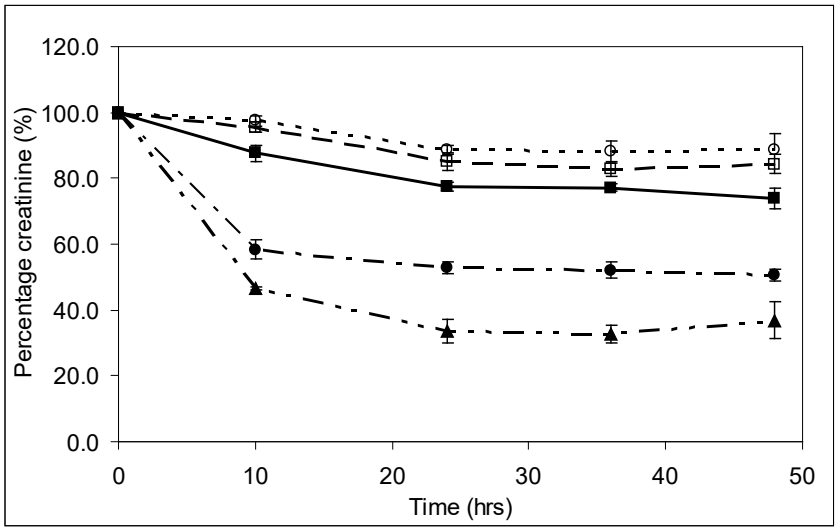

Figure 2. Effect of microcapsules on creatinine in simulated GI media over 48 hours at pH 6.4 in the proximal section of the small intestine, for a) no capsules: ○, b) empty capsules: □, microcapsules containing c) activated charcoal: ▲, d) *L. acidophilus* 314: ■, and e) activated charcoal and *L. acidophilus* 314: ●. Absolute creatinine concentrations at time zero are a) 927 µmol/L, b) 657 µmol/L, c) 600 µmol/L, d) 782 µmol/L, e) 626 µmol/L.

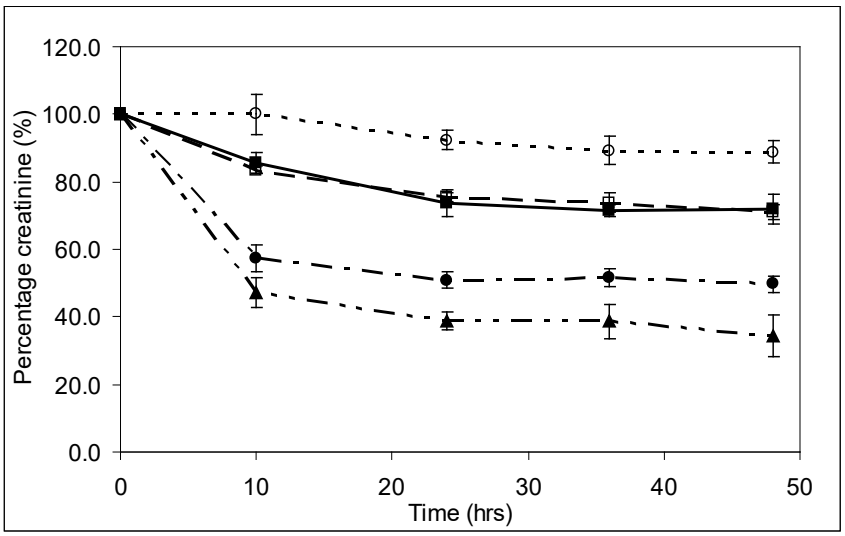

Figure 3. Creatinine profile in simulated GI media at pH 7.8 simulating conditions in the distal section of the small intestine, for a) no capsules: ○, b) empty capsules: □, microcapsules containing c) activated charcoal: ▲, d) *L. acidophilus* 314*:* ■ and e) activated charcoal and *L. acidophilus* 314*:* ●. Absolute creatinine concentrations at time zero are a) 1039 µmol/L, b) 875 µmol/L, c) 812 µmol/L, d) 883 µmol/L, e) 871 µmol/L.

pacity in the combination microcapsule is consistent with the results obtained from **Fig. 2** and may be due to obstruction of the activated charcoal pores by the probiotic.

It is important to consider that the decrease in creatinine seen at pH 6.4 contradicts data from similar studies, which concluded that creatinine concentrations are unaffected by probiotic treatment (23-25). However, those studies were done in-vivo with chronic kidney disease patients and with a combination of probiotic strains. On the other hand, this study was done in-vitro while isolating different regions of the small intestine and with a specific strain of bacteria. This suggests potential interplay both between the different regions of the small intestine as well as between different species of probiotic bacteria that may influence *L. acidophilus* 314's overall ability to metabolize creatinine. However, more research needs to be conducted on *L. acidophilus* 314's creatinine metabolic pathway and its potential influences before drawing conclusions.

Effect of microcapsules on creatinine exposure at pH 7.8 (distal section of the small intestine)

When the experiment was repeated at pH 7.8, *L. acidophilus* 314 microcapsules did not display the same ability to remove creatinine as was observed at pH 6.4; 250 µmol/L creatinine was removed after 48 hours, which was comparable to the 253 µmol/L creatinine removed by the empty microcapsules in the same period. This is because the pH range for *L. acidophilus* 314 survival is 4.0 – 6.4, therefore the slight basicity at pH 7.8 may have ceased *L. acidophilus* 314 activity. As was observed at pH 6.4, activated charcoal microcapsules showed the largest creatinine decrease of 533 µmol/L. This suggests that activated charcoal's affinity for creatinine is not pH dependent within this range. Physiologically this means that activated charcoal will function throughout the entire length of the small intestine and is only limited by saturation effects.

From **Fig. 3**, the combination microcapsules removed 438 µmol/L creatinine compared to 533 µmol/L removal by activated charcoal microcapsules despite similar amounts of activated charcoal used for both groups. This is consistent with results obtained at pH 6.4 and further suggests the physical obstruction of activated charcoal pores by the bacteria. Since this is a physical process the reduced efficacy of the combination group is not affected by pH and is observed in both regions of the SI to similar extents. The exact mechanism of the interaction between activated charcoal and *L. acidophilus* 314, as well as the metabolic process of creatinine uptake by the *L. acidophilus* 314 cells is not fully understood and further research in this area is needed.

A comparison of the creatinine removal capacities can be made from **Table 2** and supports the discussion above. Creatinine was removed at the capacities of 1.78 µmol/g activated charcoal microcapsules and 0.83 µmol/g *L. acidophilus* 314 microcapsules. The creatinine removal capacity between *L. acidophilus* 314 microcapsules and empty microcapsules (0.84 µmol/g) were comparable, indicating that *L. acidophilus* 314 did not remove creatinine at pH 7.8. Similar to pH 6.4, the combination microcapsules removed creatinine at a lower capacity compared to activated charcoal microcapsules, at 1.40 µmol/g combination microcapsules to 1.78 µmol/g activated charcoal microcapsule.

Comparing the creatinine removal capacity of 1 g microcapsules from the same test group between the two pHs, a higher removal capacity was observed at pH 7.8. This phenomenon is observed across the four microcapsule test groups. This may be due to changes in the properties of the alginate core or the chitosan membrane of the microcapsule. However, more studies are needed to examine this change in properties at different pHs.

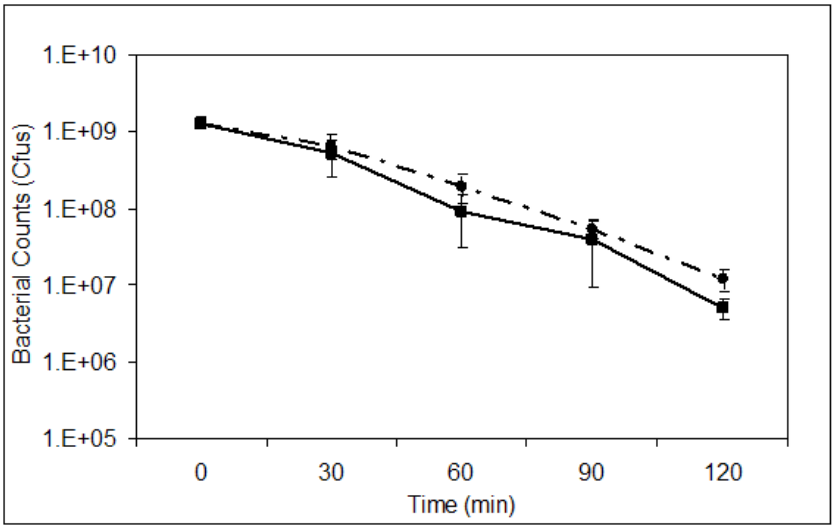

Figure 4. Viability profile of microencapsulated *Lactobacillus acidophilus* 314 in pH 1.9 simulated GI media, n=3. *Procedure:* Microcapsules containing a) *L. acidophilus* 314: ■, and b) both activated charcoal and *L. acidophilus* 314: ●, are immersed in simulated GI media pH adjusted to 1.9 at a volume ratio of 1:9 and incubated at 37 ˚C for 120 minutes. Sampling is done at 30-minute intervals, where the microcapsules were immersed in 3 % (w/v) sodium citrate for 10 min, crushed with a capsule crusher and diluted for colony plating.

Effect of activated charcoal on bacteria viability

The addition of activated charcoal to the bacteria culture did not negatively affect bacterial viability. Both *L. acidophilus* capsules and co-encapsulated capsules contained 10^9 CFU/Ml at the start of the experiment. At the end of 48 hours, microcapsules containing activated charcoal and *L. acidophilus* decreased CFU counts by one order of magnitude, while *L. acidophilus* microcapsules showed a decrease of two orders of magnitude (data not shown).

Acid tolerance test on bacteria viability

Initially, when placed in pH 1.9 acidic media simulating the GIT, both *L. acidophilus* 314 microcapsules and co-encapsulated microcapsules showed similar CFU counts. However, at the end of 120 minutes, bacterial CFU decreased 1 ½ orders of magnitude in the combination capsule, compared to a reduction of about two orders of magnitude in the *L. acidophilus* 314 capsule (Fig. 4). This small but significant difference suggests supportive functions undertaken by activated charcoal to preserve bacterial viability. Depending on food intake, the microcapsules may transit in the stomach for a maximum of 2 hours, and these results indicate the rate of decrease of bacterial viability which will help determine time and dosage administration. Although creatinine removal by the combination capsules was less effective than activated charcoal capsules, the combination capsule may be the superior choice as a result of the supportive functions of activated charcoal on the bacterial cells.

Bacterial viability in storage conditions

Results show that microcapsules containing activated charcoal preserved bacterial viability at least 5 weeks longer than microcapsules containing only bacteria; *L. acidophilus* microcapsules showed 0% bacterial viability at week 3. In contrast, the combination microcapsules showed bacterial viability until the end of week 8 when the experiment was concluded. This supports the observation that activated charcoal provides support for bacterial viability.

Upon comparison of the two storage media it is observed that the PS storage resulted in higher bacterial viability compared to the storage in 10 % urea enriched MRS broth. Comparing only *L. acidophilus* microcapsules, the CFU count for the microcapsules stored in PS was 6.7 x 10^8 CFU/mL at week 2 compared to the 1.1 x 10^7 CFU/mL CFU count for the microcapsules stored in 10 % urea enriched MRS broth. Further comparison of the combination microcapsules show that those stored in PS reflected a slower decrease of bacterial CFU counts than those in urea enriched broth; the combination microcapsules stored in PS reported a CFU count of 1.0 x10^7 CFU/mL compared to a CFU count of 2.0 x 10^3 CFU/mL for those stored in 10 % urea enriched MRS medium. This shows that the additional urea may have caused stress to the survival of the bacteria at low temperatures. As a side note, MRS broth without urea was not tested as a storage medium because of the possibility of compromising the inductive capacities of the bacteria.

Conclusion

The efficacy of creatinine removal by microcapsules containing *L. acidophilus* 314 and activated charcoal was evaluated in this study. Results show that combination microcapsules were not as efficient as the microcapsules containing activated charcoal only. However, activated charcoal in the combination microcapsule helped preserve bacterial viability during transit in media simulating the stomach, suggesting an advantage to the combination microcapsule despite its reduced efficacy. The

addition of activated charcoal also improved the bacterial viability of microcapsules, allowing them to be stored for longer and survive better in the GIT. However, due to the inconsistencies in creatinine uptake of *L. acidophilus* in the different pH environments, as well as conclusions made by other studies, more research is needed to understand creatinine's metabolic pathway in *L. acidophilus*. Regardless, this study may provide a model for the metabolic induction of different bacteria strains for the uptake of various unwanted metabolites. Microcapsules containing activated charcoal and probiotic bacteria may also potentially serve as an oral adjuvant to reduce the frequency and duration of dialysis; however, further research is still required.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Compliance

This article does not contain any studies involving human participants or animals performed by any of the authors.

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