Research Article

Oral Microencapsulated Live *Saccharomyces cerevisiae* Cells for Use in Renal Failure Uremia: Preparation and In Vivo Analysis

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Orally administrable alginate-poly-L-lysine-alginate (APA) microcapsules containing live yeast cells was investigated for use in renal failure. At all times, yeast cells remain inside the microcapsules, which are then excreted in the stool. During their gastrointestinal passage, small molecules, like urea, diffuse into the yeast microcapsules where they are hydrolyzed. Orally administrating these microcapsules to uremic rats was found to decrease urea concentrations from 7.29 ± 0.89 mmol/L to 6.12 ± 0.90 mmol/L over a treatment period of eight weeks. After stopping the treatment, the urea concentrations increased back to uremic levels of 7.64 ± 0.77 mmol/L. The analysis of creatinine concentrations averaged 39.19 ± 4.33 μmol/L, 50.83 ± 5.55 μmol/L, and 50.28 ± 7.10 μmol/L for the normal-control, uremic-control and uremic-treatment groups, respectively. While creatinine concentrations for both uremic-control and uremic-treatment groups did not differ among each other ($P > .05$), they were, however, significantly higher than those of the normal control group ($P < .05$). Uric acid concentrations averaged 80.08 ± 26.49 μmol/L, 99.92 ± 26.55 μmol/L, and 86.49 ± 28.42 μmol/L for the normal-control, uremic-control and uremic-treatment groups, respectively. There were no significant differences in both calcium and phosphate concentrations among all three groups ($P > .05$). The microbial populations of five tested types of bacteria were not substantially altered by the presence of the yeast APA encapsulated yeast ($P > .05$).

1. Introduction

In North America, renal failure is quite common, affecting approximately 5% of hospitalized patients and up to 30% of patients in intensive care units (ICUs) [1]. In 2003, 8.3 million Americans were reported to have mature chronic kidney disease with a significant number developing end stage renal disease (ESRD) [2]. Acute renal failure, or ARF, affects up to 200,000 people in the United States annually, or approximately 5% of all long-term hospitalized patients [3–5]. ARF and ESRD have high mortality characterized by an overall survival rate of approximately 50% or lower depending on the severity of the renal malfunction [6].

In most cases of renal failure, there is usually an increased retention of waste metabolites (mainly urea and creatinine) in the blood and body tissues due to a substantial decrease in urinary excretion [7–10]. As a result, blood urea nitrogen (BUN) and creatinine may increase from 18 mg/dL to 100–300 mg/dL and from 1 mg/dL to 10–25 mg/dL, respectively [11, 12]. Treating renal failure has been a persistent challenge for almost two decades. Dialysis is generally used to correct for these excess metabolites [10, 13]. However, all current renal replacement therapies (RRTs), including kidney transplantation, dialysis, and hemofiltration, display inherent disadvantages. They are characterized by serious long term complications that undermine the efficacy of these renal failure treatment modalities. Hence, new therapeutic methodologies have been investigated and developed in order to bridge the gap between efficacy and feasibility.

Among such novel potential treatments is the microencapsulation of live microorganisms capable of depleting toxic wastes and other unwanted metabolites. Previous research has demonstrated the feasibility and the potential of administrating microencapsulated micro-organisms as

an alternative oral therapy. Examples include microencapsulating genetically engineered *Escherichia coli* DH5 cells over-expressing the *Klebsiella aerogenes* urease gene for urea removal in renal failure [14], *Oxalobacter formigenes* producing oxalate-degrading enzymes for removal of accumulated oxalate in urolithiasis [15, 16].

There have been three major attempts to design novel potential alternative approaches to remove nitrogenous wastes associated with renal failure. The first uses adsorbents, such as oxystarch, to directly bind urea [10–13, 17–20]. The second method consists of hydrolyzing urea *via* co-immobilized enzyme urease and removing it *via* adsorbents [10, 12, 13, 18–20]. However, using such adsorbents requires very large doses, which is medically not advisable [11, 17, 18, 21, 22]. The third method consists of microencapsulating multienzyme systems to convert urea and ammonia into essential amino acids [13, 18, 22–24]. The problem with the last method is its poor conversion rate [23, 24].

This article investigates the potential of orally administrating APA microcapsules containing live yeast cells in renal failure uremic rates model and its feasibility to reduce the nitrogenous waste concentration levels. Towards this goal, we have recently optimized the encapsulation procedure and its respective parameters to accommodate yeast cells.

2. Materials and Methods

2.1. Chemicals. Sodium alginate (low viscosity) and poly-L-lysine hydrobromide (PLL) (*MW* 27,400) were both purchased from Sigma-Aldrich.

2.2. Microorganism and Culture Conditions. Saccharomyces cerevisiae, strain reference number 9896, was purchased from ATCC [24]. This organism is capable of producing urease urea amidolyase, which in turn metabolizes urea [25]. Yeast mold broth (YMB) growth medium was used for primary cell cultivation. YMB is composed of 10 g/L of glucose, 3 g/L of malt extract, 5 g/L of peptone and 3 g/L yeast extract. The inoculated medium was incubated aerobically at 37°C for 48 hours.

2.3. Preparation of APA Microcapsules Containing Live Yeast Cells. Log phase cultures of *Saccharomyces cerevisiae* were harvested by centrifugation at 3500 rpm for 15 minutes at 4°C. Pellets were then mixed with 2% alginate solution in order to form a final mixture consisting of 8% (v/w) pellets and 1.75% alginate. Alginate microcapsules were prepared by generating droplets of sodium alginate solution (15 mg/mL) using an IER-20 encapsulator machine manufactured by Inotech. Corp. Extruded through a 300 μm nozzle by a syringe driven pump, these droplets were gelled for 30 minutes in a well-stirred calcium chloride solution (11 mg/mL). The same droplets were then coated with a 0.1% (w/v) solution of PLL for 10 minutes to form AP beads and washed again with physiological saline (PS) for 5 minutes. The final alginate coating was then applied by immersing the beads in a 0.1% (w/v) sodium alginate solution for 10 minutes followed by a final 5-minute PS wash. The resulting

microcapsules, having an average diameter of 638 ± 17 μm, were then collected and stored in a 10/90 YMB/PS solution at 4°C. All solutions used in the preparation of these APA microcapsules were either filtered using 0.22 μm filter or autoclaved to ensure sterility. Moreover, the entire procedure was carried out in a biological hood.

2.4. Treatment Phases and Animal Monitoring. In this study, 36 (12 control normal and 24 uremic rats) male Wistar rats were purchased from Sprague Dawley Laboratory. All rats' aged between 60 and 80 days and weighted between 300 and 340 g. Both normal and uremic rats were tested upon arrival for determination of baseline urea levels (18–30 mg/dL or 3–5 mmol/L approximately for normal rats). On arrival, two rats were placed two per cage and fed rodent chow for 1 week in order to acclimatize them to the facility (sterile room with controlled temperature (22–24°C) and alternating light and dark cycles). Food and water were provided ad libitum. The animals were then be randomized in 3 groups: (1) normal control-treatment period (TP) consisting of 12 normal rats fed with alginate-ploy-L-lysine-alginate (APA) microcapsules containing live yeast cells, (2) uremic control-no-TP consisting of 12 uremic rats fed with empty APA microcapsules, and (3) uremic-TP consisting of 12 uremic rats fed with APA microcapsules containing live yeast cells.

During the experiment, the 3 groups of animals were monitored over time to evaluate changes in their urea, creatinine, phosphate, calcium, and uric acid levels. As mentioned before there was a 1 week pre-TP period to acclimatize the animals. The treatment period lasted for 8 weeks during which the rats were gavaged daily early in the afternoon and bled twice a week. The syringes were weighted in order to verify that the same amount of capsules were administered to each rat. There was a post-TP period of 2 weeks during which the animals were only bled respecting the same frequency. Animals were bled approximately 30–45 minutes post gavage.

2.5. Gavaging. 3 mL syringes (purchased from Fisher Scientific) were used to gavage the animals. Each syringe contained 2.5 mL of APA microcapsules mixed with 0.5 mL of PS. The loading was conducted in a sterile biological hood using a beaker filled with the desired preparation. Before gavaging the animals, syringes were incubated for 2 hours at 37°C in order to activated the microorganisms. The microcapsules were then orally forced-fed to the experimental rats using curved 16G stainless steel gavage needle. For this, the animal was hand-restrained and no anesthesia was used.

2.6. Blood Collection. 200 μL of saphenous blood was collected in SST microtainers (purchased from BD Biosciences) without sedating the animals. The samples were centrifuged and analyzed using a Hitachi 911 Clinical Chemistry Analyzer (Roche Diagnostics), which was loaded with the appropriate kits. Animal health indicators verification of clinical features (body weight, animal's integument, erythematic, hair coat condition, status of hydration and prorates,

animal's equilibrium: unsteadiness on legs, coordination of legs and/or abnormal reflexes, muscular disturbances, cardiovascular problems, levels of energy and motion rate, animal's respiratory system) were monitored and assessed twice a week for all the entire trial period (11 weeks).

2.7. Effect of Oral Administration of Yeast APA Microcapsules on the Gut Microbial Flora. To investigate the effect of oral administration of APA encapsulated yeast on the gut microflora, parts of rats' large intestine were obtained after sacrifice. From each group, 5 rats were chosen randomly for this purpose. The organs were homogenized mechanically until a fluid phase was obtained. Following that, 1 mL of this slurry was collected in a 1.5 mL graduated Eppendorf tube. Six 10-fold serial dilutions were performed using autoclaved PS and 0.1 mL aliquots of final dilution were plated on 5 different agar plates. The plates were then incubated aerobically according to their corresponding conditions. Bacterial enumeration for specific fecal marker microorganisms, total aerobes, total anaerobes, *Escherichia coli*, *Staphylococcus sp.* and *Lactobacillus sp.* was performed in triplicate using an agar-plate-count assay. The platting media and incubation conditions used are all listed in Table 1.

2.8. Statistical Analysis. The statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data was expressed as means ± SEM. Group and time effects were statistically investigated by ANOVA mixed models. Data were considered significant at $P < .05$.

3. Results

One of the important parameters indicating the efficiency of the treatment is the body weight, which was measured twice a week along the trial period. Figure 1 shows the average body weights of each studied group. The average body weight of the normal control group increased from 347.83 ± 11.86 g at $t = 1$ week to 464.03 ± 11.86 g at $t = 11$ weeks, respectively. Similarly, body weights for the uremic control and uremic treatment groups increased from 341.64 ± 11.86 g to 461.73 ± 29.01 g, and from 345.50 ± 9.28 g to 465.00 ± 34.56 g, respectively, between week 1 and 11 of the trial. There were significant time and group effects in rats' body weight ($P < .05$). The urea concentration, which is one of the essential indicators of the success of the proposed treatment, followed a different trend (Figure 2). Specifically, urea levels in the uremic treatment group during the pre-TP and post-TP phases were not statistically different from those for the uremic control group ($P > .05$). However, urea concentrations for the uremic treatment group in between weeks 2 and 9 were significantly different from those for the other two controls ($P < .05$). All along the 11 weeks of the trial, urea concentrations averaged 5.37 ± 0.67 mmol/L and 7.39 ± 0.99 mmol/L for the normal and uremic control groups, respectively. The average urea concentration for the uremic treatment group decreased from 7.29 ± 0.89 mmol/L to 6.97 ± 0.48 mmol/L after 1 week of treatment. During
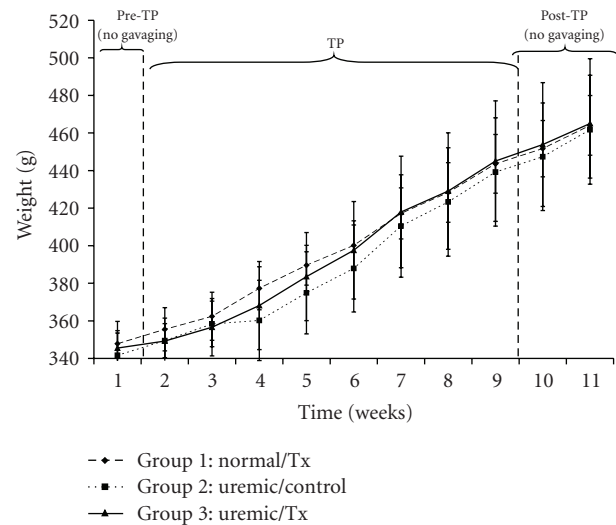

Figure 1: Rats' body weight before, during and after oral administration of APA microencapsulated yeast cells.
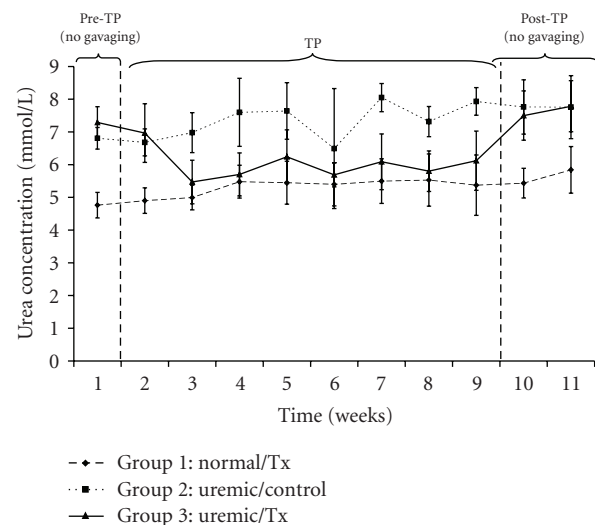

Figure 2: Plasma urea concentration before, during and after oral administration of APA microencapsulated yeast cells.

the treatment, the urea concentration for this same group averaged 6.01 ± 0.47 mmol/L before increasing again to 7.64 ± 0.77 mmol/L once the gavage had stopped.

The analysis of creatinine concentrations averaged $39.19 \pm 4.33\,\mu$mol/L, $50.83 \pm 5.55\,\mu$mol/L, and $50.28 \pm 7.10\,\mu$mol/L for the normal and uremic control as well as the uremic treatment group, respectively (Figure 3). While values for both uremic groups did not differ among each other ($P > .05$), they were, however, significantly higher than those of the normal control group ($P < .05$).

Uric acid concentrations averaged $80.08 \pm 26.49\,\mu$mol/L, $99.92 \pm 26.55\,\mu$mol/L, and $86.49 \pm 28.42\,\mu$mol/L for the normal and uremic control as well as the uremic treatment group, respectively (Figure 4). Calcium concentrations averaged 2.65 ± 0.25 mmol/L, 2.66 ± 0.15 mmol/L, and

TABLE 1: Media and incubation conditions used for enumeration of representative microbes in the human large intestine.

Microbial group	Medium	Incubation conditions and time	Colonies formed
Total aerobes	Brain heart infusion agar	Aerobic, 37C, 24 h	White
Total anaerobes	Brain heart infusion agar	Anaerobic, 37C, 72 h	White
Escherichia coli	Mc Conkey agar	Aerobic, 43C, 24 h	Red-purple
Staphylococcus sp.	Mannitol Salt agar	Aerobic, 37C, 48 h	White with yellow/purple zone
Lactobacillus sp.	Rogosa agar	Anaerobic, 37C, 72 h	White

FIGURE 3: Plasma creatinine concentration before, during and after oral administration of APA microencapsulated yeast cells.
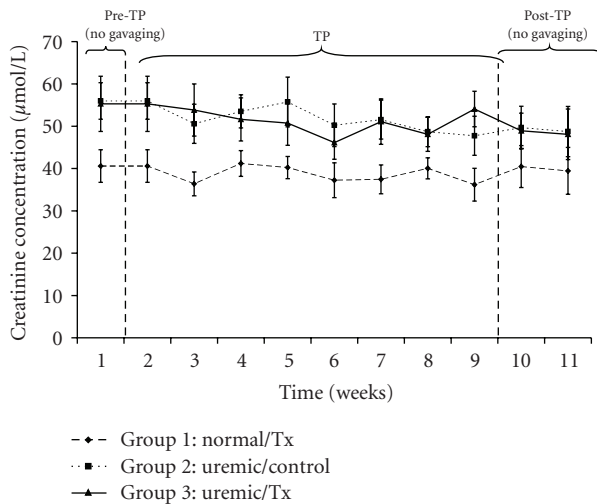

FIGURE 4: Plasma uric acid concentration before, during and after oral administration of APA microencapsulated yeast cells.

2.63 ± 0.22 mmol/L for the normal, uremic-control and uremic treatment groups, respectively (Figure 5). There were neither time nor group effects in the data for calcium concentrations ($P > .05$). Finally, phosphate concentrations, which averaged 2.84 ± 0.31 mmol/L, 2.78 ± 0.28 mmol/L, and 2.75 ± 0.33 mmol/L (Figure 6) for the normal, uremic-control and uremic treatment groups, respectively, did not display any time or group effects ($P > .05$).

An important prerequisite when orally administrating APA microcapsules containing live yeast cells is that these entities should not disturb the natural colonic gut flora. This importance is accentuated when prolonged and repeated oral intake of large quantities of microcapsules is required. Table 2 shows no marked differences in the population of the tested microbes for the uremic treatment group and those for both the normal and uremic control groups ($P > .05$).

4. Discussion

The rationale behind using an experimental uremic rat animal model stems from the striking similarities in the GI tract of rats and humans. In particular, this experimental uremic rat model has shown earlier high levels of plasma urea and other unwanted metabolites compared to normal rats, with a similar experimental endpoint. With 12 animals in each group, differences in mean uremic metabolites could be determined with a power larger than 80% and an alpha
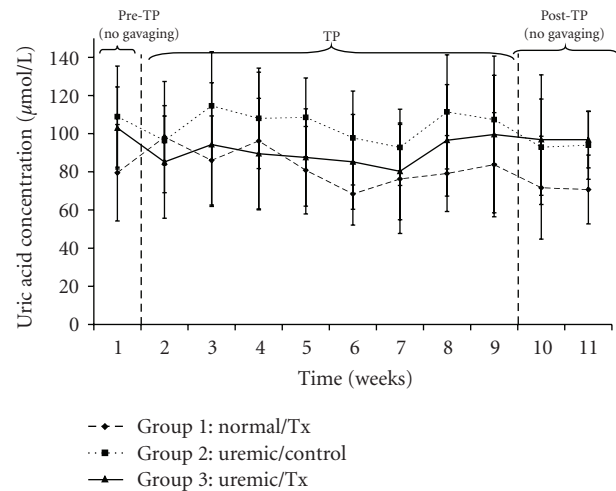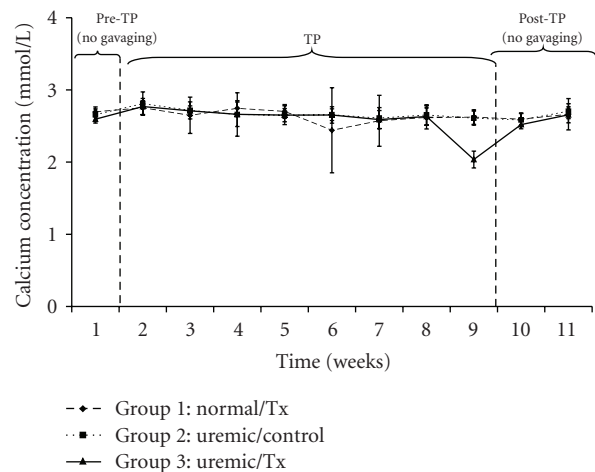

FIGURE 5: Plasma calcium concentration before, during and after oral administration of APA microencapsulated yeast cells.

error of 5%. In other words, using 36 animals allows us to detect small differences, if any exist, and, thus, infer properly about the feasibility of the treatment.

The major objective of this animal study was to determine the efficacy of microencapsulated live saccharomyces cerevisiae cells as a treatment option for lowering toxic metabolites associated with renal failure. For the first time,

TABLE 2: Effect of APA microcapsules on selected microbes in the human large intestine.

| Microbes	Incubation time [hr]	Log CFU/ml medium		
		Normal-TP	Uremic-empty APA microcapsules	Uremic-TP
Total aerobes	0	8.22 ± 0.12	8.22 ± 0.11	8.22 ± 0.09
	6	8.27 ± 0.01	8.17 ± 0.16	8.26 ± 0.08
	12	8.24 ± 0.14	8.34 ± 0.04	8.31 ± 0.07
	24	8.29 ± 0.1	8.32 ± 0.02	8.28 ± 0.03
Total anaerobes	0	8.37 ± 0.03	8.37 ± 0.01	8.37 ± 0.01
	6	8.35 ± 0.09	8.39 ± 0.02	8.31 ± 0.1
	12	8.38 ± 0.11	8.41 ± 0.07	8.38 ± 0.12
	24	8.37 ± 0.14	8.4 ± 0.11	8.36 ± 0.16
Escherichia coli	0	8.29 ± 0.13	8.29 ± 0.08	8.29 ± 0.11
	6	8.24 ± 0.16	8.33 ± 0.05	8.29 ± 0.12
	12	8.27 ± 0.11	8.24 ± 0.17	8.32 ± 0.1
	24	8.31 ± 0.07	8.28 ± 0.12	8.27 ± 0.11
Staphylococcus sp.	0	6.65 ± 0.14	6.65 ± 0.12	6.65 ± 0.1
	6	6.74 ± 0.07	6.57 ± 0.16	6.72 ± 0.03
	12	6.59 ± 0.15	6.61 ± 0.06	6.69 ± 0.01
	24	6.64 ± 0.13	6.62 ± 0.12	6.58 ± 0.08
	48	6.71 ± 0.07	6.74 ± 0.12	6.6 ± 0.17
Lactobacillus sp.	0	5.32 ± 0.01	5.32 ± 0.06	5.32 ± 0.03
	6	5.4 ± 0.05	5.41 ± 0.01	5.36 ± 0.08
	12	5.45 ± 0.03	5.39 ± 0.05	5.33 ± 0.11
	24	5.39 ± 0.09	5.37 ± 0.12	5.37 ± 0.11
	48	5.36 ± 0.1	5.35 ± 0.08	5.41 ± 0.01
	72	5.38 ± 0.17	5.42 ± 0.12	5.45 ± 0.15
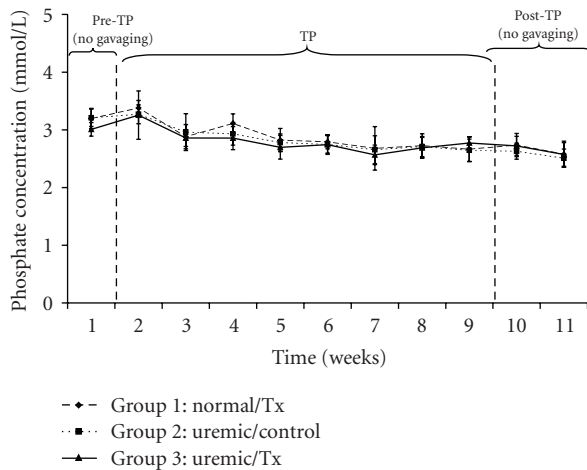
FIGURE 6: Plasma phosphate concentration before, during and after oral administration of APA microencapsulated yeast cells.

we report the use of polymeric membrane artificial cells [26–29] containing live yeast cells in renal failure. Our studies show that live yeast cells can be microencapsulated and retained inside the artificial cells during passage down the GI tract. During passage through the intestine, urea molecules diffuse throughout the permeable membrane of the artificial cells. This is rendered possible due to the molecular-weight-cut-off (MWCO) of these artificial cells. In particular, the MWCO of the alginic membrane is estimated to be between 60–70 kDa [30], while the approximate molecular weight of urea and urease are 60 kDa and 483 kDa, respectively. Theoretically speaking, urease should always stay entrapped inside the cell. Once inside the cell, urea molecules get hydrolyzed due to yeast cells, which use urea as their carbon source. The same reasoning applies for the other metabolites.

In humans, normal urea concentration ranges approximately between 3–5 ± 0.5 mmol/L (or 18–30 mg/dL) in plasma. Anything above this range is considered uremic. The urea concentrations of the normal and uremic control groups are expected to be within and higher than the normal urea range of 3–5 ± 0.5 mmol/L (Figure 2). Noting that the urea concentrations of the uremic treatment group decreased at the start of the treatment and increased to uremic values after stopping the treatment suggests that the APA encapsulated yeast show efficacy and work as hypothesized. By analyzing the data, one can notice that urea in uremic treated rats decreased by approximately 18% from the pre-TP phase level. During the treatment 8-week treatment period, the urea concentrations for the uremic-TP group stagnated around 6.01 ± 0.47 mmol/L, which is around 1.1 times greater than the average urea concentration of the normal control group. Upon terminating the oral administration of the APA encapsulated yeast, the plasma urea level returned rapidly to the uremic values (Figure 2). This means that the artificial

cells were all excreted in the stool and were not retained in the intestinal tract.

The concentrations of creatinine and uric acid (Figures 3 and 4) did not vary significantly within and among each uremic group but were significantly different from those of the control group. Specifically, the creatinine concentrations of uremic treated and not treated groups were 1.29 and 1.28 times greater than those of the normal group, respectively. Similarly, those of uric acid for the same uremic groups were 1.23 and 1.07 times greater than those of the normal group, respectively. These trends suggests that the orally administered APA encapsulated yeast did not have any effect on lowering neither creatinine nor uric acid, which are both correlated with decreased renal function [31, 32]. Similarly, the artificial cells did not influence calcium ($P > .05$) and phosphate ($P > .05$) plasma electrolytes because there was no variation in their concentrations neither with respect to time nor among groups (Figures 5 and 6).

Body weight, which is an indication of the overall health condition, increased throughout the trial period (Figure 1). This suggests that the animals maintained a constant growth. Furthermore, each rats' group displayed body weight values that are statistically different from the other two groups with respect to time. However, renal failure individuals have usually their body weight decreasing with time. If the TP is proven successful, we would have expected the body weight of the uremic control rats to decrease and that of the normal and uremic-TP groups to increase. Specifically, the body weight of the normal and uremic controls increased by $33.41 \pm 2.1\%$ and $35.15 \pm 1.9\%$, respectively, at the end of the 11th week. Similarly, the uremic-TP had their body weight increased by $34.59 \pm 0.9\%$. During the TP period, the increase in the body weight of the uremic-TP group is significantly larger than that of the uremic control group. This fact is not surprising and suggests that the treatment is somehow effective in alleviating some of the uremia effects. However, the only possible explanation to the rise in the body weight of the uremic control rats is that the decrease in this parameter due to uremia is outweighed by its increase due to growth.

It is known that a well-balanced gut microbiota plays a crucial role on the *ensemble* of the human health [33]; upsetting the intestinal flora may lead to complications and colonizations/infections [34]. The results from the *in vivo* viability study (Table 2) could suggest that the materials used to produce the microcapsules combined with the yeast itself did not evoke any appreciable adverse effects on the simulated human intestinal flora.

5. Conclusion

Normally, the kidneys are responsible for removing metabolic waste metabolites and maintaining water and electrolyte balance. In the case of renal failure, these tasks cannot be fulfilled by the damaged kidneys and patients need alternative treatment to survive. Dialysis and kidney transplantation are considered effective but expensive. Other alternatives such as the use of absorbents are challenged with the need of large doses of absorbents [10–13, 17–20]. This work presents a novel yeast-based approach to treat renal failure uremia. Oral administration of these microcapsules to uremic rats was found to decrease urea levels by $18 \pm 0.31\%$ over a period of eight weeks. After stopping the treatment, the urea concentration increased back to the uremic values. However, this formulation had no perceivable effects on the concentrations of creatinine, uric acid, phosphate, and calcium. The microbial populations of the five tested types of bacteria were not substantially altered by the presence of the APA encapsulated yeast. This suggets, in consequence, that there were no perceivable adverse effects of oral administration of these capsules on the microbial flora of the human GI tract.

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