DEVELOPMENT OF A CALCIUM-FORTIFIED DRINK BASED ON KEFIR, EGGSHELL, CITRIC FRUITS, AND TAP WATER

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ABSTRACT

Calcium is an essential and critical component of human health. However, most people do not cover daily calcium recommendations. Therefore, a calcium-based drink, called BEVERAGE, is developed in order to offer an alternative source of this mineral to the population. A combination of kefir, eggshell, citrus fruits, and tap water were investigated. Orange was selected because it provides the highest amount of carbohydrates for fermentation, and it is accessible to the population. The proportion of components that produce the highest concentration of calcium in the BEVERAGE was with 10 g of kefir, 6 g of eggshell, 200 ml of orange juice, and 800 ml of tap water, 20 °C, and 72 h of incubation. With these conditions, the BEVERAGE was fortified with 600 mg calcium/liter. In addition, the intestinal absorption of calcium was evaluated through an ex vivo model of the everted small intestinal sacs, proving to be higher than the control solutions (water+CaCl2, juice+CaCl2). This study provides a methodology to prepare at home a beverage that could contribute to increase daily calcium intake.

Keywords: calcium deficiency, eggshell, kefir grains, calcium supplementation

INTRODUCTION

Calcium is an essential and critical component of human health. This element takes part in various biological processes, such as nerve conduction, muscle contraction, bone structure, signal transduction, regulation of hormonal secretion, and vascular activities (Li et al., 2018). About ninety-nine percent of the calcium contained in the human body is stored in the skeleton under the form of hydroxyapatite and other calcium salts. Calcium intake is of utmost importance during the early stages of life, since the increase in bone mass takes place up to the age of 30 years old (Wiodarek et al., 2014).

Although calcium is abundant in foods, its bioavailability is very different among them. Many plant-based foods have low bioavailability of calcium, especially due to the presence of oxalic acid (Etchevery et al., 2012). Conversely, dairy products are excellent sources of calcium and the bioavailability is high, favoured by the presence of carbohydrates (Cámara-Martos & Amaro-López, 2002), milk proteins (Shimizu, 2004) and peptides generated from the digestion of proteins of milk (Li et al., 2018). The most important source of calcium is dairy products, constituting more than 60 % of daily intake (Gonnelli et al., 2009). The intake of calcium from other sources can reach a maximum of 250 mg Ca/day. For these reasons, the consumption of dairy products is recommended for the adequate development of bone mass (Reid et al., 2014). In the prevention of osteoporosis, besides the consumption of dairy products, calcium supplements are available to compensate for the deficiency in the intake of this micronutrient (Heaney, 2000).

The Recommended Dietary Allowance (RDA) of calcium set by Institute of Medicine for adult people is 1000 mg. In general, a minority of individuals reaches these goals (Fiacioni et al., 2020), often by self-diagnosis of the disease (Nicklas et al., 2011). In addition, the number of consumers following a vegan diet has markedly increased, and their influence in the food sector will likely continue to grow (Rosenfeld, 2018). Concerns like dietary fat, cholesterol, and other related allergies, among some individuals, have led to switch their preferences from dairy to non-dairy products. Consequently, these people are at risk of low calcium intake. Thus, the difference between recommended and actual calcium intake compel the manufacturers to market an increasing number and variety of calcium-fortified products (Konar et al., 2015).

Food fortification is one of the most effective strategies to improve nutrient intake. Fortification has been used in high-income countries for more than 80 years. However, despite this vast experience, fortified foods are not always accessible to low socioeconomic groups (Allen et al., 2006). However, outside of dairy and these beverages, there are few calcium-rich foods for non-dairy consumers, most of which contain less calcium per serving than dairy products and dairy substitutes (Hodges et al., 2019).

The composition of the chicken eggshell and its use as a low-cost source of calcium has been evaluated, showing that 1 g of chicken eggshell could provide 50 % of an adult's daily requirement (Brun et al., 2013). Because it is possible to eliminate potential pathogens on eggshells, eggshell consumption as a calcium supplement can be considered safe and could play a role in relieving calcium deficiency (Bartter et al., 2018). The chicken eggshell has been used to fortify milk with calcium simultaneously with the use of microorganisms (kefir). The fermentative activity of kefir produces a decrease in pH, which favours the dissolution of the eggshell, which is composed mainly of calcium carbonate (Fina et al., 2016). On the other hand, water kefir is a fermented and home-produced beverage made by adding kefir grain to sugar solution in water and incubating this mixture at 20-25 °C for at least 12 h, and then separating of kefir grain to other production. Fresh or dried fruit slices can be added for flavour and removed at the end of the fermentation period (Muneer Alsayadi et al., 2016).

Given the importance of calcium for the organism and knowing the low number of individuals to reach the RDA of calcium for different reasons, the objectives of our study were to develop a high calcium content drink based on kefir, eggshell, citrus fruits, and tap water, and we refer to this preparation as the “BEVERAGE”. It will be useful if the calcium is absorbed at the intestinal level. For this reason, the following objective was to evaluate the intestinal calcium absorption in rats.

MATERIAL AND METHODS

Fruit selection

Juices from citrus fruits were tested, due to easy handling for its extraction, its natural acidity, and its accessibility. To select fruit for fortification, juices from tangerine, orange, grapefruit, and lemon were obtained and pH, titratable acidity, conductivity, and carbohydrates (glucose and fructose) content were measured.
Optimization of BEVERAGE preparation

The incorporation of calcium to the BEVERAGE was obtained with kefir, eggshell, orange juice, and tap water. Kefir was obtained from a commercial brand of Argentina (Gepsa, BA). Dry eggshells were mechanically processed using a mixer mill (Retsch GmbH, MM200, Haan, Germany) for 20 min at a frequency of 30 s-1 as a standard method. Then the chicken eggshell powder was sterilized in an automatic sterilizer autoclave (Microclave SL 9000, Buenos Aires, Argentina) for 15 min at 134 °C and 30 psi (according to manufacturer's instructions) before adding to the BEVERAGE. Different levels of factors were tested to reach the greatest dissolution of the CaCO₃ from the eggshell. A factorial design was carried out using the following factors and their respective levels: eggshell quantity (0, 2.5, 4, and 6 g), temperature (0, 24, 48 and 72 h) and temperature (8, 12 and 20 °C). All samples had 10 g of kefir, 200 ml of orange juice and 800 ml of tap water. Since it is a full factorial design, the number of experimental units arises from the product of the number of levels of each factor. Calcium concentration of tap water mean=15.4 mg/L, SD=5.3, n=30. At the end of the fermentation process, calcium and pH were measured.

Intestinal calcium absorption

The experiments that are described from here were carried out with the following composition: kefir 10 g, eggshell 6 g, orange juice 200 ml, tap water to reach 1000 ml, fermented at 20 °C for 72 h. See the below results that support this decision. The intestinal calcium absorption is a process that mainly occurs in the small intestine (Díaz De Barboza et al., 2015). Calcium intestinal absorption was evaluated through an *ex vivo* model of the everted small intestinal sacs (Brun et al., 2009). Experiments were carried out in female Sprague Dawley rats of 180–220 g of body weight. Rats, provided by the School of Medicine, Rosario National University (Argentina), were fed with balanced food for rodents (Gepsa, Argentina) and water ad libitum. Before the experiments, rats were kept in a temperature-controlled environment of 23-25 °C, with a 12h-12h light-dark cycle and filtered airflow at scheduled time interval. All the experiments were conducted in accordance with international guidelines for animal care (Ollert et al., 1993) and this work has been approved by the Ethical Committee of the School of Medicine, Rosario National University (number of resolution: 2162/2017). Preparation of everted small intestinal sacs: after euthanasia by CO₂ inhalation, the small intestine was removed from 4 rats and everted, rinsed with 9 g/L NaCl solution, and divided into segments of similar size. The serous surfaces of the everted small intestinal sacs were exposed for 30 minutes to 0.5 ml of the following solution, which is called internal solution: 1 mmol/L Tris, 1 mmol/L Mgl₂, 160 mmol/L glucose, 100 mmol/L beta-glycerophosphate, and pH 9. This solution does not contain calcium, and the final concentration of calcium is used to calculate calcium absorption. The mucous surfaces were exposed to different solutions: 

- **BEVERAGE** (n=14 sacs), prepared as stated before.
- Water+CaCl₂ (n=14 sacs), CaCl₂.
- Juice+CaCl₂ (n=14 sacs), orange juice and CaCl₂.

Analytical grade calcium chloride was used as a control because it has a high bioavailability (> 90 %), it is soluble in water and neutral effect on taste and colour of the product (Tiwari et al., 2018). All solutions were prepared and were adjusted to reach the following values: 

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>80.51±29.58</td>
</tr>
<tr>
<td>Fructose</td>
<td>148.29±72.98</td>
</tr>
<tr>
<td>pH</td>
<td>2.27±0.19</td>
</tr>
<tr>
<td>Conductivity</td>
<td>4.10±1.03</td>
</tr>
<tr>
<td>Titratable acidity</td>
<td>555.22±275.62</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>42.76±21.84</td>
</tr>
</tbody>
</table>

The effect of the different factors (time, temperature, amount of eggshell, amount of kefir) on the calcium concentration of the BEVERAGE was analysed with a factorial design and multiple analyses of variance. Then, the individual effect of each level of the different factors was analysed with the coefficient of a linear model. A previous analysis rejected the interaction between factors. According to the ANOVA, the amounts of eggshell and fermentation time are factors that significantly influence calcium concentration (p<0.05). The incubation time 72 h and 6 g of eggshell contributed the highest calcium content, providing 600 mg/L. The following linear model was used: calcium concentration=a*amount of eggshell + b*temperature + c*time + intercept, where a, b and c are coefficients of the model. The model fitted experimental values (R²=0.91, p<0.05) and the amount of eggshell (2.5, 4, and 6 g) and the time (24, 48, and 72 h) are factors that significantly influence calcium concentration (p<0.05), but not temperature (Tab 2). The model assigns coefficient equal to zero for the lowest value of each factor (reference factor). A positive coefficient indicates an increase in calcium concentration compared to the reference level of each other. The effect of the different time, temperature, and amount of eggshell on calcium concentration was analysed through a linear model and PCA. According to the linear model, the amount of eggshell and the time were factors that significantly influence calcium concentration of BEVERAGE. The temperature has not effect on calcium concentration. According to linear model, the optimal conditions that allow a drink with a greater amount of calcium were: 6 g of the eggshell with fermentation for 72 h, providing 600 mg Ca/L. This concentration was achieved with a small amount of carbohydrates available for fermentation. This suggests that the higher the amount of carbohydrates, the higher the calcium concentration is likely to be achieved. More studies are needed to test this hypothesis. 

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**Table 1** Measured variables in citrus fruits

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Lemon</th>
<th>Grapefruit</th>
<th>Orange</th>
<th>Tangerine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.27±0.19</td>
<td>3.09±0.38</td>
<td>3.66±0.29</td>
<td>3.89±0.36</td>
</tr>
<tr>
<td>Conductivity</td>
<td>4.10±1.03</td>
<td>2.91±0.27</td>
<td>2.87±0.76</td>
<td>2.39±0.14</td>
</tr>
<tr>
<td>Titratable acidity</td>
<td>555.22±275.62</td>
<td>220.24±58.74</td>
<td>109.48±43.61</td>
<td>90.55±39.66</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>42.76±21.84</td>
<td>80.51±29.58</td>
<td>148.29±72.98</td>
<td>144.23±62.91</td>
</tr>
</tbody>
</table>

Measured variables expressed as mean ± standard deviation. Different letters between columns of the same row indicate significant differences between solutions. ANOVA, LSD test (p<0.05).

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Optimization of BEVERAGE preparation

The effect of the different factors (time, temperature, amount of eggshell, amount of kefir) on the calcium concentration of the BEVERAGE was analysed with a factorial design and multiple analyses of variance. Then, the individual effect of each level of the different factors was analysed with the coefficient of a linear model. A previous analysis rejected the interaction between factors. According to the ANOVA, the amounts of eggshell and fermentation time are factors that significantly influence calcium concentration (p<0.05). The incubation time 72 h and 6 g of eggshell contributed the highest calcium content, providing 600 mg/L. The following linear model was used: calcium concentration=a*amount of eggshell + b*temperature + c*time + intercept, where a, b and c are coefficients of the model. The model fitted experimental values (R²=0.91, p<0.05) and the amount of eggshell (2.5, 4, and 6 g) and the time (24, 48, and 72 h) are factors that significantly influence calcium concentration (p<0.05), but not temperature (Tab 2). The model assigns coefficient equal to zero for the lowest value of each factor (reference factor). A positive coefficient indicates an increase in calcium concentration compared to the reference level of each other. The effect of the different time, temperature, and amount of eggshell on calcium concentration was analysed through a linear model and PCA. According to the linear model, the amount of eggshell and the time were factors that significantly influence calcium concentration of BEVERAGE. The temperature has not effect on calcium concentration. According to linear model, the optimal conditions that allow a drink with a greater amount of calcium were: 6 g of the eggshell with fermentation for 72 h, providing 600 mg Ca/L. This concentration was achieved with a small amount of carbohydrates available for fermentation. This suggests that the higher the amount of carbohydrates, the higher the calcium concentration is likely to be achieved. More studies are needed to test this hypothesis.
Calcium concentration and pH had a significantly correlation ($R^2$=0.94, p<0.05). As an attempt to find the relationship between calcium concentration and pH in the presence of other variables, Principal Components Analysis (PCA) was used. The analysis of data with PCA analysis confirms those found with a linear model. PCA analysis can be found as Complementary analysis.

Table 2 Coefficient of the linear model proposed for each factor

<table>
<thead>
<tr>
<th>Factor levels</th>
<th>Coefficients (mg/L)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggshell: 0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Eggshell: 0.5 g</td>
<td>56.16</td>
<td>0.001867</td>
</tr>
<tr>
<td>Eggshell: 2.5 g</td>
<td>238.95</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>Eggshell: 4 g</td>
<td>355.79</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>Eggshell: 6 g</td>
<td>463.96</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>Temperature: 8°C</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Temperature: 12°C</td>
<td>13.89</td>
<td>0.387171</td>
</tr>
<tr>
<td>Temperature: 20°C</td>
<td>16.06</td>
<td>0.317904</td>
</tr>
<tr>
<td>Time: 0 h</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Time: 24 h</td>
<td>43.87</td>
<td>0.017572</td>
</tr>
<tr>
<td>Time: 48 h</td>
<td>82.80</td>
<td>1.89e-05</td>
</tr>
<tr>
<td>Time: 72 h</td>
<td>120.97</td>
<td>4.25e-09</td>
</tr>
</tbody>
</table>

P-value <0.01 indicates significant difference in calcium concentration from the reference level of the factor.

Although plant-based food contains inhibitor factors to calcium absorption, microbial fermentation is one of the actual methods to obtain bioactive nutrients. During the fermentation process, microorganisms transform complex substances into small ones that improve the nutritional quality of foods (Xiang et al., 2019).

Intestinal calcium absorption

The calcium absorption rate of the BEVERAGE was evaluated in everted small intestinal sac model. The BEVERAGE had a higher absorption rate than the juice+CaCl₂ and even than the water+CaCl₂ solution, ANOVA, LSD test, p<0.05 (Tab 3).

Table 3 Calcium absorption rate of ex vivo small intestinal everted sac after 30 min incubation with different mucus solutions

<table>
<thead>
<tr>
<th>Mucus solutions</th>
<th>Calcium absorption rate (µg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water+CaCl₂ (n=14)</td>
<td>20.5±2.3⁴</td>
</tr>
<tr>
<td>juice+CaCl₂ (n=14)</td>
<td>9.2±4.1⁹</td>
</tr>
<tr>
<td>BEVERAGE (n=14)</td>
<td>32.8±6.3⁹</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation. Different superscript letter indicates significant differences. One way ANOVA, LSD test (p<0.05).

Studying calcium absorption through an ex vivo model contributes to better control of the experiments and a greater number of experimental units. Furthermore, the everted small intestinal sac technique allows the reduction of animals because control animals from other experiments can be used. Instead, the isolated intestinal model implies the use of 1 live animals per experiment. However, with the model used in this paper, it is not possible to evaluate the calcium absorption taking into account the integrity of the system.

Mineral waters rich in calcium have been reported to be a valuable and bioavailable source of Ca with beneficial effects measured through biomarkers and bone densitometric parameters (Vannucci et al., 2018). Also, intestinal calcium absorption from the BEVERAGE was significantly higher than the juice+CaCl₂. This difference could be explained because of the presence of citrate and other substances present in the juice, which has a complex action with calcium. Kefir could metabolize these acids, avoiding the formation of calcium with low absorption. However, this hypothesis should be verified.

Figure 1 Principal Component Analysis (PCA).

PCA describes the effect of time, pH, temperature, and the amount of eggshell on the concentration of Ca. Figure 1 shows map variables of PCA indicating that pH and calcium concentration are positively correlated, as indicated by the direction of vectors that represent the variables. The map also indicates that the samples with higher pH and calcium concentration are preferentially placed in the right half-plane, while samples with lower pH and calcium concentration will be most probably found in the left half-plane. In addition, an analysis of the ellipses was carried out, which shows that longer incubation time and quantity of eggshell allow higher values of calcium concentration and pH.

Figure 2 displays the samples and the 0.95 confidence ellipses of different incubation time. The higher the incubation time, the higher the pH and calcium concentration. Ellipses display the position of samples with different incubation times. Although there is no great differences, the ellipses indicate that the higher the incubation time, the highest the calcium concentration. Figure 3 displays the effect of temperature on calcium and pH. The results indicate that the temperature is the principal factor to control in the preparation process of the BEVERAGE. Figure 4 displays samples and 0.95 confidence ellipses for a different amount of eggshell, showing higher pH and calcium concentration as the amount of eggshell increases.

Figure 3 displays the samples and the 0.95 confidence ellipses of different incubation times. Although there is no great differences, the ellipses indicate that the higher the incubation time, the highest the calcium concentration. Figure 3 displays the effect of temperature on calcium and pH. The results indicate that the temperature is the principal factor to control in the preparation process of the BEVERAGE. Figure 4 displays samples and 0.95 confidence ellipses for a different amount of eggshell, showing higher pH and calcium concentration as the amount of eggshell increases.

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Figure 2 Upper panel. Incubation time. ⊙: 0 h, △: 24 h, ◊: 48 h, ○: 72 h. Lower panel. 95% confidence ellipses for different amount of eggshell. Shadow right area has high calcium concentration and high pH values.
Although calcium is an essential and critical component for human health, the vast majority of people do not cover the daily recommendations. Dairy products are the primary source of calcium, but many individuals cannot or do not choose to consume them. The development of new sources of calcium that allow a drink to be consumed instead of milk is an area of growing interest.

**CONCLUSION**

The results of this job indicate that the BEVERAGE is a safe, practical, and acceptable method to improve dietary calcium intakes. The BEVERAGE has certain characteristics that deserve to be highlighted: high calcium bioavailability, the possibility of home preparation, and use of household waste. Besides, the BEVERAGE does not contain milk, which makes it useful for those people who for various reasons cannot consume dairy products.

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**Conflict of Interest:** Maria Eugenia Chulibert, Cecilia Casabonne, Silvana Sandra Ramadán, and Alfredo Rigalli declare that they have no conflict of interest.

**REFERENCES**


**Figure 3** Upper panel. Incubation temperature. ○: 8 °C, △: 12 °C, +: 20 °C. Lower panel: 95 confidence ellipses for different amount of eggshell. Shadow right area has high calcium concentration and high pH values.

**Figure 4** Upper panel. Amount of eggshell. ○: 0 g, △: 0.5 g, +: 2.5 g, ◊: 4 g, x: 6 g. Lower panel: 95 confidence ellipses for different amount of eggshell. Shadow right area has high calcium concentration and high pH values.


