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Comparative Study of Probiotic Properties of *Lactobacillus Casei* M3PM99 and *Lactiplantibacillus Plantarum* M1PM99 Isolated from Iranian Traditional Cheese

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ABSTRACT

Introduction: This article aims to compare the in vitro probiotic potential between *Lactobacillus casei* and *Lactiplantibacillus plantarum* isolated from Semnan traditional cheese.

Methods: The bacteria isolated from traditional cheese were sequenced based on the 16s rRNA gene and confirmed as *L. casei* and *L.plantarum* in the BLAST database. The probiotic properties were assessed in terms of resistance and growth in gastric juice and bile salts, survival rate in simulated gastrointestinal conditions, antimicrobial effect, hydrophobic properties and auto-aggregation and co-aggregation capabilities.

Results: The Survival rate of *L. plantarum* (72.3%) was higher than *L. casei* (68%), exhibiting higher growth rates at pH levels of 3.5 and 4.5., *L.plantarum* demonstrated superior growth rate across various bile concentrations (0.3, 0.5 and 1%) (P <0.05). *L. casei* was more resistant to simulated intestinal condition than *L. plantarum*. The antimicrobial spot method revealed that *L. casei had* a stronger antagonistic effect against *Staphylococcus areus,* while *L. plantarum* showed greater efficacy against *E. coli*. The well diffusion test confirmed the results of the previous antimicrobial method against these pathogens. Both lactobacillus isolates exhibited high percentage of auto-aggregation after 5 hours of incubation, ranging from 91.16% to 94.48%. Regarding co-aggregation, *L. plantarum* (42/02%) showed a significantly higher percentage than *L. casei* (21.02%) than co-aggregation with *S.aureus* (p<0.05). The hydrophobic properties of *L.Casei* (18.34%) were significantly lower than those of L.plantarum (57.62%) (P<0.05).

Conclusion: The results indicate that *L. plantarum* isolated from traditional cheese is a promising candidate for further probiotic studies.

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Introduction

World Health Organization and Food and Agriculture Organization have defined Probiotics as “live micro-organisms that, when administered in adequate amounts, confer a health benefit to their host” (1).

Lactic acid bacteria (LAB) represent one of the most important groups of probiotics, which include various beneficial strains, particularly those in the lactobacillus genus (2). Probiotic Lactobacilli are recognized for their health-promoting effects, including the prevention of pathogenic colonization, the stimulation and maturation of host tissue. LAB play crucial roles in anti-pathogenic activities, such as production of antimicrobial compounds including bacteriocins, hydrogen peroxide and diacetyl (3), as well as enhancing epithelial barriers and regulating immune system (4). Due to these beneficial properties, lactobacillus strains have gained widespread attention and are primarily used as probiotics (5). LAB strains are found in different dairy and non-dairy food products, such as yogurts, cheese, fruit juices and fermented sausages. Additionally, these bacteria are considered part of the natural flora in the digestive tract, mouth and urinary-genital tract of humans and animals (6). *L.Plantarum* recognized as a safe probiotic, plays a significant role in both the food industry and human medicine by regulating immune system, reducing the risk of tumors and cholesterol levels and maintaining a balanced intestinal flora (7).

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L.casei is a gram-positive mesophilic, obligate homofermentative, microaerophilic, catalase-negative, spore-free bacterium known for its high capacity for acid production (8). Traditional fermented foods exhibit a rich diversity of LAB as part of their natural microflora, identifying native strains of LAB in traditional dairy products, that are compatible with and adapted to specific regions can significantly improve health outcomes (9). Khiki cheese is a popular traditional fermented foods in Semnan province, Iran. However, there has been limited research comparing the probiotic properties of the two strains, *L. plantarum* and *L.casei,* isolated from this traditional cheese. The present study aims to compare the probiotic potential of *L. plantarum* and *L.casei* isolated from Semnan traditional khaki cheese, focusing on their resistance and growth in gastric juice and bile salts, survival rates in simulated gastrointestinal conditions, antimicrobial effects, hydrophobic properties and auto-aggregation and co-aggregation capabilities.

Materials and Methods

In this research, lactobacillus strains which were isolated from Semnan traditional cheese were utilized by the Department of Food hygiene and Quality control, Faculty of Veterinary Medicine, Semnan University. The isolated bacteria were sequenced based on the 16s rRNA gene and confirmed as *Lactobacillus casei and Lactiplantibacillus plantarum* in the Basic Local Alignment Search Tool (BLAST) database. These strains were registered as new enties in the National Center for Biotechnology Information (NCBI) gene bank and codes M3PM99 and M1PM99 respectively. The isolates were further confirmed through biochemical and molecular tests (sequencing) and were stored at -80° C. Man-Rogosa-Sharpe (MRS) Broth, MRS agar, and Brain-Heart Infusion (BHI) agar culture media were used to cultivate and enumerate the studied bacteria. These media were prepared according to the manufacturer's instructions and sterilized using an autoclave (Merck, Germany).

Viability and Growth in Acidic pH

To determine the resistance of the selected Lactobacillus isolates to acidic pH, MRS broth was adjusted to pH levels of 1/5 and 2/5. A 10µl aliquot of a 24-hour culture of the isolates (10^8 CFU / ml) was mixed with 240µl of the adjusted MRS broth in a microwell plate and incubated for 3 hours at 37 ° C. Serial dilutions were prepared at 0 and 3 hours, followed by surface plating on MRS agar medium. The plates were then incubated under aerobic conditions (24- 48 hours) at 37 ° C. Then bacterial count revealed survival percentage of the strains. Additionally, growth potential in acidic medium was assessed in MRS broth at different pH levels (2/5, 3/5 and 4/5) based on preliminary screening tests. Growth curves were generated by OD values which were obtained at 630 nm, measured using an ELISA reader (Biotech, USA) over a 24-hour period (10).

Bile salt Tolerance and Growth

Both strains were selected for a bile tolerance test in MRS broth containing 0/3%, 0/5% and 1% bile salts. Growth curves were drawn using OD readings at 630 nm over 24 hours period measured with an ELISA reader (Biotech, USA).

Simulation of Tolerance to the Gastrointestinal Tract

Bacterial cultures (18 hour) were centrifuged at 6000× g for 5 min and washed twice with Phosphate-Buffered saline (PBS). Then, suspended in PBS solutions (pH 2), which included pepsin (3 mg/ml) and NaCl (0.5%) and incubated at 37 ° C. Finally, colonies were counted on MRS agar at 0, 60, 90 and 180 minutes. To evaluate survival in simulated intestinal condition, 1 ml of the previous culture was mixed with 9 ml of intestinal juice, which consisted of 1 mg/ml of pancreatin and 0.3% bile at pH 8. Live bacteria were counted at 0, 2 and 4 hours (11).

Antibacterial Activity

Antimicrobial effects of both isolated strains were investigated using spot and well diffusion methods against *staphylococcus aureus ATCC 29213* (gram-positive indicator) and *Escherichia coli ATCC 35218* (gram-negative indicator). For the spot agar test, a 24-hour culture of both lactobacillus strains was spotted onto the surface of MRS agar and incubated for 24 hours at 30° C. Five milliliters of BHI agar was mixed with 100µL of the indicator microorganisms (equivalent to 0.5 MCFarland turbidity standard) and poured over the spot-inoculated MRS agar. The plates were incubated at 35 ° C, and the diameter (mm) of growth inhibition zone around each spot was measured to assess antagonist activity. In the well diffusion test, 15ml of BHI agar was mixed with 100µL of the indicator microorganisms (equivalent 0.5 MCFarland turbidity standard) and poured into a plate. After the agar solidified, wells with a diameter of 6mm were punched into the agar using a sterile Pasteur pipette. The bottom of each well was sealed with melted agar, and then 100µL of cell-free supernatant was added to each well. The plates were incubated at 35° C for 24 hours. Finally, the diameters of the growth inhibition zone (mm) were measured.

Hydrophobicity of the Cell Surface of Bacteria

Hydrophobicity of Lactobacillus strains was assessed by investigating their potential of adhering to hydrocarbons such as xylene, toluene and n-hexadecane. Lactic acid bacteria isolates were incubated at 37°C for 24 hours and then centrifuged. Then cells were washed twice by using PBS and suspended in 3 ml of 0.1 M KNO3. The optical density at 600 nm (A_0) was record. Three milliliters of the cell suspension were mixed with 1ml of hydrocarbon (xylene) for separation. After a 10- minutes Pre-incubation at room temperature, the final two-phase system was mixed by vortex for 2 minutes. The aqueous and organic phase were then allowed to separate for 20 minutes at room temperature. The aqueous phase at the bottom was collected, and its optical density at 600 nm (A_1) was measured. Hydrophobicity was calculated using the following formula:

$$H (\%) = (1-(A_1/A_0)) \times 100$$

Auto Aggregation and Co-Aggregation Test

The auto-aggregation property of isolated strains can influence the binding capacity of bacterial cells to intestinal epithelial cells, while the co-aggregation property is related to the prevention of pathogens colonization in the intestine (12). To determine auto-aggregation, 4 mL of the 1-day culture which contained 10^8 CFU / ml of bacteria, was vortexed for 10 seconds and then incubated at room temperature for 5 hours. At hourly intervals, 0/1ml suspension was removed and placed in another tube containing 3.9 ml of PBS. The absorption at 600 nm was then measured using a spectrophotometer. Auto-aggregation was calculated using the following formula:

$$Auto\text{-}aggregation\ (\%) = 1-(A_t/A_0) \times 100$$

A_t represents the absorbance at 1, 2, 3, 4, and 5 hours, A_0 is the absorbance at time 0.

Co-aggregation was measured by mixing equal volumes (500 µL) of both strains and another microorganism (either LAB or pathogens such as *Staphylococcus aureus* and *Salmonella typhimurium*). The suspensions were incubated at room temperature for 5 hours. The absorbance of the mixed and single cultures was measured at 600 nm. Co-aggregation was calculated using the formula:

$$Co\text{-}aggregation\ (\%) = [(A_{pa} + A_{pr})/2 - (A_{mix})] / A_{pa} + A_{pr} /2 \times 100$$

Where A_{pa} Represents the optical absorbance value of pathogenic bacteria, A_{pr} represents the optical absorbance value of probiotics, and A_{mix} represents the optical absorbance value of the mixture of probiotics and pathogenic bacteria (Gregorian et al., 2017).

Statistical Analysis

All measurements were performed in triplicate and analyzed by SPSS-20 software and obtained mean values were statistically compared by using the Duncan test at a 95% confidence level ($p < 0.05$).

Results and Discussion

Viability and Growth in Acidic pH

Results of acidic tolerance of both isolated strains in vitro situation indicated that none of them grew at pH 1.5, while both survived significantly at pH 2.5 (Table 1). Additionally, the study demonstrated a significant difference (72.3%) in the survival rates of L.*plantarum* and *L.casei,* with *L.plantarum* displaying a higher survival rate *(p<0.05).*

Table 1. The results of survival rate of Lactobacillus isolates at pH 2.5 at 0 and 3 hours after incubation.

Strains	$ph = 2.5\ \ log/ml$		Survival
	H0	H3	
L. plantarum	8.17Log/ml	Log/ml 5.91	72.3%
L. casei	Log/ml 8.08	5.5Log/ml	68%

Investigations on the functional properties of probiotics in simulated gastric conditions showed that Lactobacillus isolates could grow at vary pH levels (2.5, 3.5 and 4.5). The growth rate of the control group was significantly higher at pH 4.5 (P <0.05) but no significant difference was observed at the other pH levels. Furthermore, the growth slope of both strains at pH 2.5 was comparable, however, at pH levels 3.5 and 4.5, the growth rate of *L.plantarum* exceeded that of *L.casei (P‹0.05).* It is expected that potential probiotic strains can tolerate acidic pH levels, due to the production of more than 2 liters of gastric juice with acidic pH from the cell lining the stomach each day, this creates an effective natural barrier with high acid content against the entry of live bacteria into the digestive tract. Tabatabai Yazdi et al., 2015, investigated the effect of pH on the survival of 55 isolates obtained from kimchi, Finding that less than 50% of most Lactobacillus strains could survive at pH 2(13). These findings align with our results and those from previous studies, which indicated that Lactobacillus strains exhibit a loss of viability at lower pH levels. for instance, Sahadeva et al. (2011) reported that the viability of all isolates significantly decreased at pH 2 compared to pH 3 and 7(14). In 2014, Hashemi et al. studied on the Lactobacillus strains which were isolated from traditional Kurdish cheese and tested their probiotic characteristics. Their results indicated that Lactobacillus strains survived exposure to pH levels of 2 and 3 for 2 hours. However, It is important to note that fermented products like Kurdish cheese can increase gastric pH, thereby aiding in the survival of these strains (10).

Bile Salt Tolerance and Growth

Results showed that cell wall structure, external coat characteristics and lipid composition of lactobacilli were affected by bile medium which resulted to significant changes of surface properties (15). Consequently, bile salt tolerance of probiotics is considered as an important property to be able to exert their beneficial effects in this biological fluid. According to obtained results, both isolated strains could resist and grow at various concentrations of bile adequately. While the treatment group of *L.plantarum* did not have significant difference, but *L.casei* displayed a considerably higher growth rate at 0.3% bile compared to 0.5 and 1% concentrations. Furthermore, the highest growth rate across different bile concentrations was attributed to L.*plantarum* (P <0.05). Bile salts are biological substances that possess strong antimicrobial activity by disrupting the lipid bilayer and integral proteins of cell membranes, as well as triggering DNA damage (14,15). Common responses to bile stress in *Lactobacillus spp.* include activation of molecular machinery, utilization of bile efflux systems and the action of bile salt hydrolases.(15). In the study conducted by Farhangfar et al in 2021, 22 *Lactobacillus plantarum* strains were isolated from Siahmazgi traditional cheese, and their probiotic properties was evaluated. The results showed that 10 out of the 22 strains could grow at low pH levela. In further investigations, 5 out of 10 strains demonstrated significant growth during hours of exposure to 0.5% bile salt, although they also exhibited resistance to 0.15% bile at the same time(16). Another study by Hassanzadeazar in 2012, identified *L.plantarum* and *L.casei* as the most resistant strains isolated from Koozeh cheese at a 0.3% bile concentration(17).

The high resistance of some bacteria to low pH may be due to the production of compounds such as polysaccharides that prevent the effect of acid on their cell membranes. It has been shown that bile tolerance by probiotics is bile- and strain-specific, with resistance levels ranging from 0.125 to 2% bile concentrations (16).

Simulation of Tolerance to the Gastrointestinal Tract

Probiotic strains should be able to survive in the gastrointestinal tract in order to exert their positive effects on the host adequately. The gastrointestinal environment exerts a severe stress on probiotics, so they should tolerate this situation and survive in high concentrations in this medium. In simultaneous gastrointestinal medium, the concentration of viable cells decreased after three hours. However, the results indicated a good survival rate of both selected isolates, specifically, in the gastric simulation, approximately 1.31 log CFU g^{-1} decrease was observed. In the intestinal simulation, not only did the isolates no reduction in colony numbers, but an increase was also observed, likely conferring probiotic benefits. (Table2).

Table2. Survival results of Lactobacillus isolates in simulated the gastrointestinal tract

Strians	$ph = 3\ \ log\ cfu/ml$		$ph = 8\ \ log\ cfu/ml$	
	H_0	H_3	H_0	H_4
L. casei	8	6.47	7.17	7.88
L. plantarum	7.6	6.36	7.3	7.8

L.casei demonstrated greater resistance in the simulated gastrointestinal tract compared to *L.plantarum*. Probiotics must be capable to overcoming the acidic gastric environment to reach and colonize the small intestine of the host (18). Studies suggest that in food supplement which are enriched by probiotics, minimum concentration of active cells in ileum portion of the small intestine should be 6 log CFU g−1. However, it should be noted that laboratory and in vitro conditions cannot fully replicate all mechanical kinetic, and chemical physiological conditions of the digestive system (in vivo). Due to the production of compounds such as polysaccharides, some probiotic bacteria exhibit high resistance to gastric juice. In line with recent findings, Sarabi Jamab et al. in 2017 investigate the survival rates of probiotic bacteria, such as *Lactobacillus acidophilus*, *Lactobacillus rhamnus* and *Bifidiobacterium bifidium*, at lower gastric pH (1.5) compared to higher pH (2.5). Their results indicated that survival rates were significantly lower at pH 1.5. Additionally, when *Bifidiobacterium bifidium* enters the intestine, its colonies are fewer compared to other times in the intestine environment. The highest number of *Lactobacillus acidophilus* was observed after 30 minutes in the intestine. (11).

Antibacterial Activity

One of the most important characteristics of probiotic strains, is their antimicrobial activity. The results from the spotted assay method revealed that the highest antagonistic effect against *S.areus* and *E.coli* was exhibited by *L.casei* and *L. plantarum,* respectively. Results from the well diffusion test indicated that the growth-inhibition zone for *L.casei* against *S.areus* was larger than that for *L.plantarum.* (Table 3) Conversely, the larger inhibition zones around *E. coli* were attributed to *L.plantarum* (Figure 1).

Table 3. The mean results of Antimicrobial spotted and well diffusion assay methods

Strain	*S.areus*		*E.coli*	
	Spot assay	Well diffusion	Spot assay	Well diffusion
L.plantarum	2.37±0.05	0.84±0.01	2.40±0.38	1.42±0.08
L.casei	2.87±0.92	1.35±0.05	2.07±0.37	1.25±0.01

Lactobacilli have been reported to exhibit significant antimicrobial activity, primarily due to their ability to excrete antimicrobial substances such as organic acids, hydrogen peroxide (H_2O_2), and bacteriocins. This characteristic inhibits the growth of a broad range of pathogens (19). Sung-Min Lim et al. in 2020 investigated the antimicrobial activity of *L.plantarum* isolated from fermented foods against *Streptococcus mutans* and found that this strain could potentially be used as a biofilm inhibitor and oral probiotic in functional foods (20). According to Hashemi et al., 2014, all lactobacillus strains showed significant resistance against the two studied pathogens, however there was no antagonistic activity observed against against *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus.* Notably, there was a significant difference between probiotic strains in their ability to inhibit pathogenic bacteria (10). In a similar study, Bromberg et al. 2004, reported that the antimicrobial activity of the 61% of LAB isolated from fermented meat products was only 31%(21).

Hydrophobicity of the Cell Surface of Bacteria

Hydrophobicity is a property which may help probiotic strains to survive and colonize in gastrointestinal medium due to competing for host cell binding sites (22). Thus, probiotic strains should be able to survive and colonize the gastrointestinal tract (23). According to the results, the hydrophobicity (%) of *L.Casei* (18.34±0.21) was significantly lower than the *L.plantarum* (57.62±0.22) (P<0.05). Cell adhesion is entirely dependent on membrane proteins and cell surface features (24). In the study by Hashemi et al. (2014), the percentage of hydrophobicity ranged from 43.6% to 82.8%. Additionally, This study revealed that *L.plantarum LP3* exhibited the highest hydrophobicity among reference strains(10). Similar results were reported in a study by Anwar Abdullah et al. (2014), who evaluated the hydrophobicity of six LAB strains, finding percentages ranging from 29.5 to 77.4 (25). In another study, involving 51 Lactobacillus isolates from Brazilian cheese, *L.plantarumbrevis SAU105* demonstrated the highest hydrophobicity ability (60.98%) (26).

Low and high affinity indicates the surface of bacterial cells is hydrophilic and hydrophobic respectively. Based on previous studies, the hydrophobicity of strains could vary due to different medium and suspension composition or variety of surface-linked proteins among strains. For example, presence of some groups such as glycol proteins on the cell surface result to higher hydrophobicity, while a hydrophilic surface mostly is related to presence of polysaccharides (27).

Auto Aggregation and Co-Aggregation Tests

auto-aggregation, or self-clumping is an important characteristic of probiotics that promote colonization of the human intestine, prevent pathogen infections and modulates the colonic mucosa. Therefore, these strains can inhibit adhering of pathogens to intestinal mucosa (28). The mean auto-aggregation value after 5 hours incubation at 37°C ranged from 91.16% to 94.48%. However, there was no significant differences in auto-aggregation percentages between the Lactobacillus isolates. Co-aggregation is another potential mechanism for antagonistic activity against pathogen. This property represents a probiotic strain's ability to inhibit undesirable bacteria and prevent surface colonization by pathogenic microorganisms. The co-aggregation of Lactobacillus strains with pathogens was also investigated in this study. Results were represented by comparing absorbance of the mixed and individual suspensions after 4 hours (Figure2).

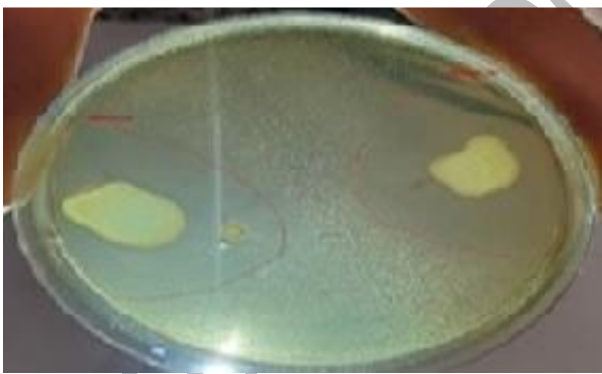

Figure 1. Results of Antimicrobial spotted well diffusion well diffusion method of *L.plantarum* against *E.coli*

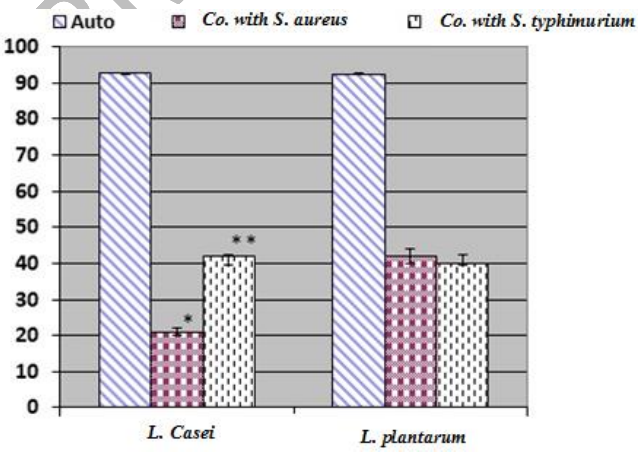

Figure2. Examination of co-aggregation and auto-aggregation of the studied strains

Regarding the co-aggregation feature of the studied strains, *L.plantarum* (42/02%) showed a significantly higher co-aggregation percentage than *L.casei* (21.02%) with *S.aureus* ($p<0.05$). In the study by Hashemi et al. (2014), all Lactobacillus strains isolated from traditional Kurdish cheese exhibited a high percentage of auto-aggregation, with results ranging frpm

77.3% to 80.2% after 4 hours of incubation. The co-aggregation results with *E. coli* and *S.aureus*, were significantly related to the *L.plantarum* strain *AF₁* (29.2%)(10). These reports confirmed the results of our study. According to Talebi et al. the *Enterococcus* isolate 7C37 demonstrated the highest co-aggregation effect (18.9%) against three pathogenic bacteria, along with high auto-aggregation (18.9%), which align with the results of this study(29). In another study, three strains including *Lactobacillus acidophilus MR-1980*, *Lactobacillus heloticus INRA-2010-H11* and *Lactobacillus acidophilus JM-2010* from 25 LAB strains which were isolated from dairy products and fermented vegetables, exhibited auto-aggregation values of 53.3 and 70.5 and 62.3 respectively (30). The aggregation ability of probiotic bacteria refers to their capacity to adhere to intestinal epithelial cells. This feature is regarded as an essential property of probiotics for influencing the immune system and biofilm formation in the gastrointestinal tract. According to Tuo et al. (2013), the adhering ability (auto-aggregating, co-aggregating, hydrophobicity) of LAB strains within the same species showed a positive correlation(31). This suggests that these properties are likely due to the presence of surface-bound proteins and other macromolecules that played a partial role in the adhering and auto-aggregation abilities of the strains, thus, these mechanisms are dependent on genus, species and strain (32).

Conclusion

Lactiplantiacillus plantarum strain which was isolated from Khikki traditional Semnan cheese had considerable probiotic properties in vitro. Therefore, it can be suggested as a new probiotic strain to food industry and may be considered for further in vitro and in vivo studies to determine potential health benefits.

Declarations

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Conflict of Interest

The authors report no conflicts of interest.

Authors Contributions

Conceptualization, M.P..; methodology, M.P and N.G.; software, A.J.J and A.S.; formal analysis, A.J.J.; investigation, N.G.; data curation, N.G.; writing—original draft preparation, F.E.; writing—review and editing, M.P and A.S.

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