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OPTIMIZING THE MIXING FORMULAS OF CORDYCEPS TEA: USING SENSORY METHOD AND TESTING HARMFUL MICROORGANISM

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SUMMARY

This study aimed to develop a raw material blending process and evaluate the sensory characteristics of an herbal tea mixture containing *Cordyceps militaris* in combination with pineapple (*Ananas comosus*), ginger (*Zingiber officinale* (Willd.)), lemongrass (*Cymbopogon citratus*), and chrysanthemum (*Chrysanthemum indicum*). The research showed no presence (negative) of harmful microorganisms and quantities that could affect consumers in two consecutive tests. The sterilization and raw material blending process met the food safety standards (TCVN). The sensory evaluation results from consumers identified two mixed herbal (NT2 and NT5) tea formulations that received the highest ratings (7.730 and 8.040 points, respectively) and they were significantly higher than the remaining formulations while being not significantly different from each other, indicated that researchers can select suitable raw materials and blending ratios for large-scale production.

Keywords: *Cordyceps militaris*, pineapple (*Ananas comosus*), ginger (*Zingiber officinale* (Willd.)), lemongrass (*Cymbopogon citratus*), chrysanthemum (*Chrysanthemum indicum*), herbal tea formulation.

INTRODUCTION

Cordyceps militaris, a rare and valuable fungus, has long been applied in traditional medicine for the improvement of health and various ailments (Zhou *et al.*, 2009). Research by Oh and colleagues highlights that *Cordyceps militaris* contains several beneficial components, such as cordycepin, polysaccharides, and steroids, which boost the immune system, reduce inflammation, and energize the body (Oh *et al.*, 2019a; Oh *et al.*, 2019b).

Despite its numerous health benefits, the everyday application of *Cordyceps militaris* encounters significant challenges, primarily due to its high cost and the scarcity of pure products. To make Cordyceps more accessible, one viable approach is integrating it with other natural ingredients in daily consumables like tea.

Pineapple (*Ananas comosus*), ginger (*Zingiber officinale* (Willd.)), lemongrass (*Cymbopogon citratus*), and chrysanthemum (*Chrysanthemum indicum*) globally recognized for their culinary and health-promoting properties. Ginger, with its anti-inflammatory and analgesic properties (Mashhadi *et al.*, 2013), offers a warm, spicy counterbalance to Cordyceps' bitterness. Pineapple provides bromelain, a digestive aid (Maurer, 2001), and introduces a sweet, tangy flavor that complements the strong taste of Cordyceps. Lemongrass, known for its refreshing taste, helps mitigate the bitterness of Cordyceps while offering stress relief. Chrysanthemum, commonly used in herbal teas, provides a soothing flavor and benefits such as stress reduction and sleep quality enhancement (Adib-Hajbaghery, Mousavi, 2017).

However, there is a scarcity of comprehensive research on combining these specific ingredients into a tea blend that is both flavorful and health-promoting. Hence, this study seeks to fill this gap by developing a tea formula that incorporates *Cordyceps militaris* with these selected ingredients. The goal is to evaluate this blend's flavor, ascertain its health benefits, and solicit consumer feedback, thereby contributing valuable insights into food and medical product innovation.

MATERIALS AND METHODS

Materials

Cordyceps militaris, commonly known as Nhong trung thao in Vietnamese, was sourced from the Ho Chi Minh University of Industry and Trade (HUIT) and selected based on biological maturity. The *Cordyceps militaris* used had to be fresh, free from mold, intact, and without antibacterial or chemical odors.

Raw materials were chosen based on their known health benefits and availability. For instance, pineapples were procured from Long An Province, a region known for producing the best-quality pineapples in Vietnam due to its high enzyme content. The pineapples used were ripe but not overly so, free from disease or damage, and possessed the characteristic aroma of fresh pineapples. The selected pineapple must have a Brix index ranging from 12 to 14, ensuring natural sweetness and fresh flavor. Simultaneously, the pineapple's pH level is checked to ensure it falls within the range of 3.5 to 4.0, aiding in maintaining the stability of the tea blend.

Fresh ginger was harvested from Quang Nam Province due to its superior essential oil. It had to be fresh, mold-free, without signs of pest infestation, and had the distinctive aroma of ginger.

Lemongrass was obtained from the Tan Phu Dong District in Tien Giang Province. The lemongrass was fresh, mold-free, without signs of pest infestation, and had a mild, pleasant aroma.

Dried chrysanthemum flowers, commonly used as an ingredient in herbal products or foods, were supplied by BV Pharma Joint Stock Company.

All raw materials underwent rigorous inspection before use to ensure they met the quality standards and were free from harmful toxins or pathogenic bacteria. Selection criteria were based on quality, flavor, and health benefits. *Cordyceps*, lemongrass, and ginger were dried at 50-60°C until they became crisp and moisture-free. Chrysanthemum flowers were naturally sun-dried in a well-ventilated area, avoiding direct sunlight.

The blending process was conducted at 25°C with a humidity level of 50% to ensure optimal preservation of the ingredients' sensory and health properties. Drying was performed using a standardized food dehydrator at 40°C for 12 hours to achieve consistent dryness across all samples.

Survey of mixing ratios

Cordyceps militaris, pineapple, ginger, lemongrass, and chrysanthemum were tested in various mixing ratios, and the sensory evaluation of the resulting tea infusion was conducted based on color, aroma, and taste (Stone and Sidel, 1973a).

Fractional Factorial Experimental Design Method (Montgomery, 2012) was employed to optimize the model for assessing the influence of various factors. *Cordyceps*, pineapple, ginger, lemongrass, and chrysanthemum were mixed according to the standard procedure, and microbial counts were conducted at each stage. Figure 1 illustrates the fractional factorial design used in this study, which systematically varied five key ingredients across different levels to identify their impact on sensory appeal and health benefits

Collecting consumer feedback

To evaluate the flavor and health benefits of *Cordyceps* tea based on consumer feedback, a standardized sensory test was conducted (Stone and Sidel, 1973b), following these steps:

(1) Participant selection: A random group of consumers was selected.

(2) Experiment introduction: A brief introduction was provided to the participants regarding the purpose of the experiment and how to participate.

(3) *Cordyceps* tea tasting: Each participant was served *Cordyceps* tea and asked to taste it, providing feedback.

(4) Scoring: The Hedonic scale was used to collect feedback from the participants (Pimentel *et al.*, 2016):

9 - Like Extremely
8 - Like Very Much
7 - Like Moderately
6 - Like Slightly
5 - Neither Like nor Dislike
4 - Dislike Slightly
3 - Dislike Moderately
2 - Dislike Very Much
1 - Dislike Extremely

(5) Data Analysis:

We chose the Hedonic scale because it directly and intuitively reflects consumers' liking or disliking of the product, serving as an effective and popular sensory evaluation tool in food science. While its main advantage is the quick and direct assessment of emotions, a limitation is its inability to identify specific reasons behind each reaction and potential bias due to participants' subjectivity.

The collected data were synthesized and analyzed to determine how the flavor of *Cordyceps* tea was rated based on the Hedonic scale, optimal mixing ratios, and other relevant experiment-related evaluations.

Microbial indicators

Microbial indicators were analyzed according to TCVN standards, including total anaerobic bacteria according to TCVN 4884:2008, *Salmonella* according to TCVN 4829:2005, *E. coli* according to ISO 9308-1:2014, and *Coliform* according to TCVN 6848:2007.

Data processing

Microsoft Excel 2016 and the JASP Statistics Program running on the Windows platform were used to analyze the collected data.

RESULTS AND DISCUSSION

Experimental layout and application of fractional factorial design method

In terms of basic theory, the total number of complete experimental formulations (full factorial) would be 1 (*Cordyceps*) x 3 (Pineapple) x 3 (Ginger) x 3 (Lemongrass) x 3 (Chrysanthemum) = 81 experimental formulations. However, a full factorial experimental design could face time, raw materials, and cost limitations. Therefore, this research applied the fractional factorial experimental design method. Instead of using three levels for each ingredient, the study used the two lowest and highest levels. Thus, with four types of ingredients (pineapple, ginger, lemongrass, chrysanthemum) and two levels of blending weight, we would have $2^4 = 16$ experimental formulations (EF).

Reducing from a full design of 2^4 (16 experiments) to half the design (8 experiments) is called a half-fractional factorial design. In this case, the interactions of 3 or 4 factors are assumed to be insignificant. For example, it could be assumed that the interaction between pineapple, ginger, and lemongrass (PGL) is insignificant**.**

The design's degree of freedom (df) is 24 - 1 = 23, and there are four main factors, 6 two-factor interactions, four three-factor interactions, and 1 four-factor interaction. When assuming that one three-factor interaction is not significant, it can be used to estimate another effect, thus reducing the number of required experiments. Based on this assumption, the reduced eight experiments are designed according to table 1.

Blending of ingredients and evaluation of microbiological criteria

The washing process is meticulously executed by rinsing the raw ingredients for exactly 5 minutes under clear water at a temperature of 25°C, ensuring the effective removal of surface contaminants. The water flow rate is maintained at 2 liters per minute to achieve optimal cleansing without eroding the quality of the ingredients. The ingredients are processed and blended following the standard procedures outlined in figure 1. At each stage, the microbiological content is tested according to TCVN standards, including total anaerobic bacteria per TCVN 4884:2008, *Salmonella* per TCVN 4829:2005, *E. coli* per ISO 9308-1:2014, and *Coliform* per TCVN 6848:2007.

Table 1. Experiment design.

TN/NL	Fractional Factorial Design				Half-Fractional Factorial Design			
	P.	G.	L.	C.	P.	G.	L.	C.
TN1	D1	G1	S1	C1	D1	G1	S1	C1
TN2	D1	G1	S1	C3	D1	G1	S3	C3
TN3	D1	G1	S3	C1	D1	G3	S1	C3
TN4	D1	G1	S3	C3	D1	G3	S3	C1
TN5	D1	G3	S1	C1	D3	G1	S1	C3
TN6	D1	G3	S1	C3	D3	G1	S3	C1
TN7	D1	G3	S3	C1	D3	G3	S1	C1
TN8	D1	G3	S3	C3	D3	G3	S3	C3
TN9	D3	G1	S1	C1				
TN10	D3	G1	S1	C3				
TN11	D3	G1	S3	C1				
TN12	D3	G1	S3	C3				
TN13	D3	G3	S1	C1				
TN14	D3	G3	S1	C3				
TN15	D3	G3	S3	C1				
TN16	D3	G3	S3	C3				

P, G, L, and C: Pineapple, ginger, Lemon grass and chrysanthemum

1 and 3: Mixing weight 0.5g and 1.5g

1 g of *Cordyceps* was added per experiment

Assumption of insignificant factors was based on assessment demand and adjustable. In these experiements, assuming that the interaction among P, G, and L is insignificant.

Testing microorganisms in a sample

The pour plate method was used after incubating the sample at 37 ± 1°C for 24-48 hours to determine the total anaerobic bacteria count. The bacterial count in 1 mL of the test sample was determined by counting the colonies grown on agar plates from various dilution levels.

The *Salmonella* identification method typically involves the following steps: enrichment, selective enrichment, isolation, and confirmation. In the enrichment step, 25 mL of the sample is transferred to a sterile PE bag and supplemented with 225 mL of BPW solution. The mixture is then homogenized and incubated at 37°C. After 18-24 hours, selective enrichment is performed by transferring 0.1 mL of the enriched culture to 10 mL of Rappapport-Vassiliadis (RV) selective enrichment medium. The mixture is then incubated at 42 ± 0.2°C for 12-24 hours. Subsequently, single colonies are isolated by streaking from the selective enrichment medium onto Salmonella differential agar (MLCB).

The determination of *E. coli* most probable number (MPN) assumes the number of positive tubes in the EC medium and indole production in peptone water at 44°C corresponds to the highest probability MPN value.

The *Coliforms* criteria are applied according to TCVN 4882:2007 ISO 4831:2006. This technique uses the MPN method and employs an MPN index table to calculate the results. The total Coliform count in 1 g or 1 mL of the food product is determined by counting the positive tubes after inoculation into brilliant green lactose bile broth at 35 ± 2°C for 24-48 hours.

The results of the microbiological testing (Table 2), following TCVN standards, showed no presence (negative) of harmful microorganisms and quantities that could affect consumers in two consecutive tests. The blended product is packaged and stored under specified conditions before sensory evaluation is conducted with a selected group of individuals.

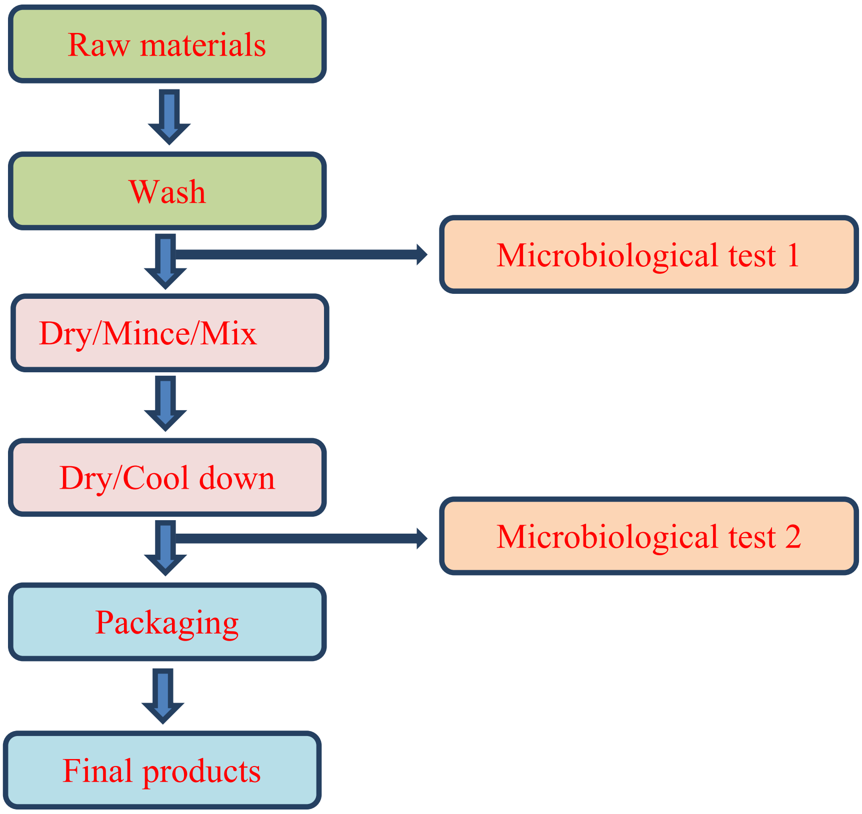

Figure 1. Ingredient blending process.

Distribution of consumer evaluation scores and the minimum sample size for the study

Each test group was evaluated by 50 consumers (400 evaluation scores from 8 test formulations). These evaluation scores need to be checked for sample distribution and objectivity. Each evaluation score is considered an independent event, and these events should follow a normal distribution law before conducting in-depth analyses.

The results of the Q-Q plot analysis in figure 2 show that most of the collected data points lie on the 45-degree diagonal line from the origin. This indicates that the consumer evaluation scores were objectively collected and followed an ordinary distribution law.

In this study, we selected 8 test formulations with 50 evaluators for each formulation using a 1 to 9 point scale. The research's effectiveness is evaluated based on the number of participants, ensuring a Power > 0.9, meaning that if the study were repeated ten times, there would be more than nine times more accurate and consistent data. Figure 3 shows that with Power > 0.9 and α error ≤ 0.5, the minimum required sample size for the study is approximately 40 individuals per group (using analysis of variance). The specific number is 44 individuals per experiment (see Appendix 1). Compared to the sample size used in this study (8 experiments x 50 individuals/experiment = 400 individuals), it is entirely appropriate.

Table 2. Microorganism count in the samples.

Test No./Count	Anaerobic bacteria count	*Salmonella*	*E. coli*	*Coliforms*
Test 1	-	-	-	-
Test 2	-	-	-	-

- None of target microorganisms (negative).

+ Contain target microorganisms (positive).

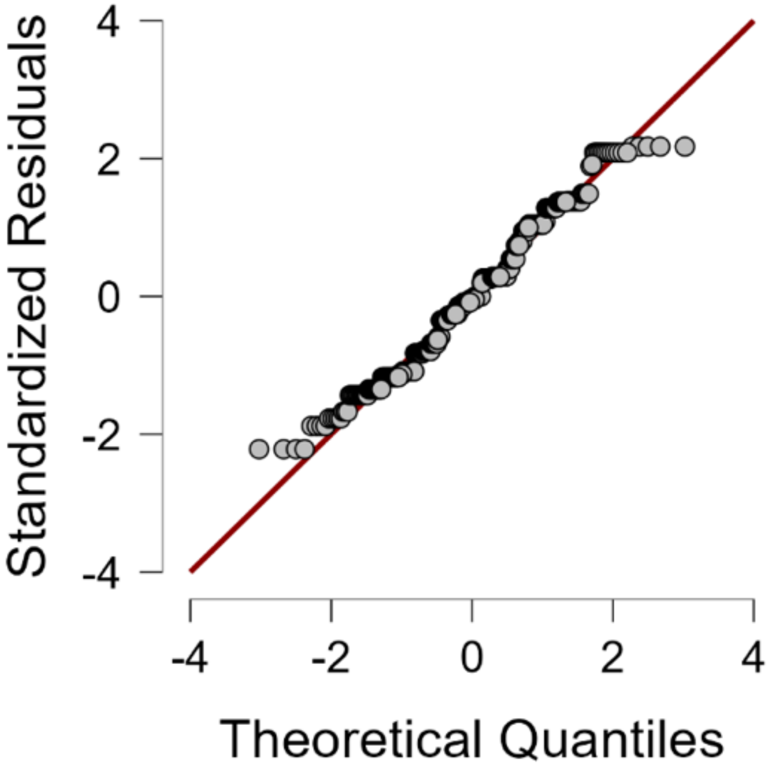

Figure 2. Distribution of sensory evaluation data score (n=400).

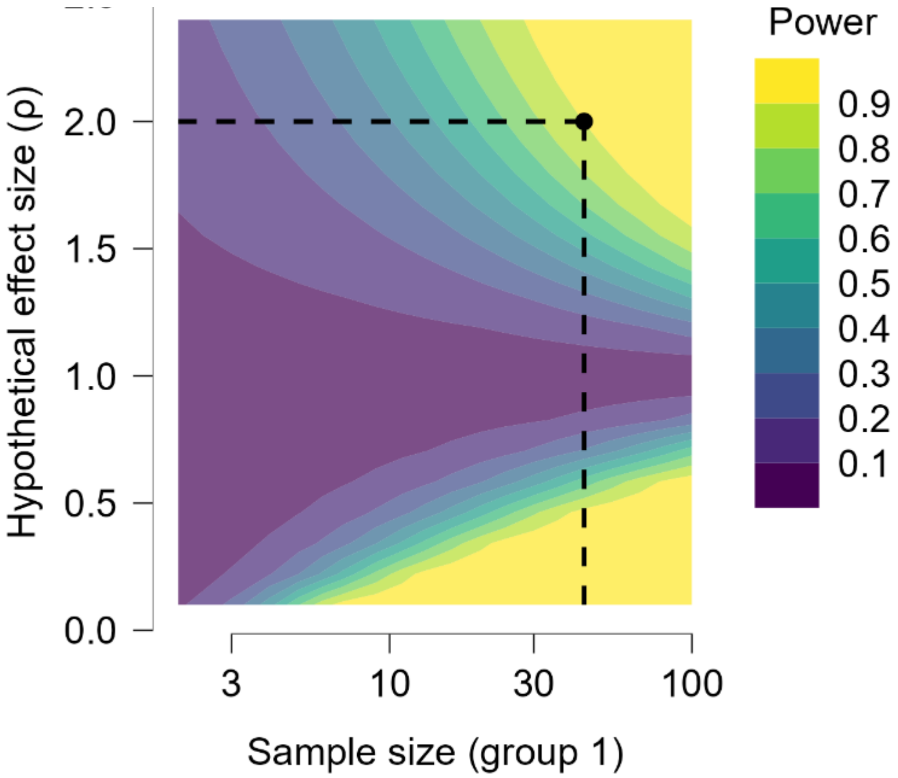

Figure 3. Study minimum sample size (power > 0.9, α ≤ 0.5).

Data analysis summary

Four hundred data points were collected, and there were no erroneous data. Evaluation scores ranged from 5 to 9 points. The Raincloud plot in figure 4 displays the data distributions for each test formulation, including mean, frequency, median, standard deviation, and confidence interval (see Appendix 2).

Test formulation 5 (TF5) had the highestmean score of 8.040 ± 1.019, while TF1 had the lowest mean score of 5.760 ± 0.600. The mean evaluation scores, sorted from lowest to highest, for the test formulations, are as follows: TF1 (5.760 ± 0.600) < TF4 (6.000 ± 0.1942) < TF8 (6.240 ± 0.876) < TF7 (6.320 ± 0.946) < TF3 (6.630 ± 0.850) < TF6 (7.080 ± 1.188) < TF2 (7.730 ± 0.910) < TF5 (8.040 ± 1.019). TF5 had the highest frequency at 9.000, while TF4 had the lowest frequency at 5.000.

Analysis of differences between test formulations

In this study, ANOVA was employed to assess whether there were statistically significant differences among the 8 test formulations. Evaluating consumer perception of each mixed formulation is of significant importance in production and business strategy planning and in substituting different formulations to achieve changes in taste without substantially altering the consumer perception.

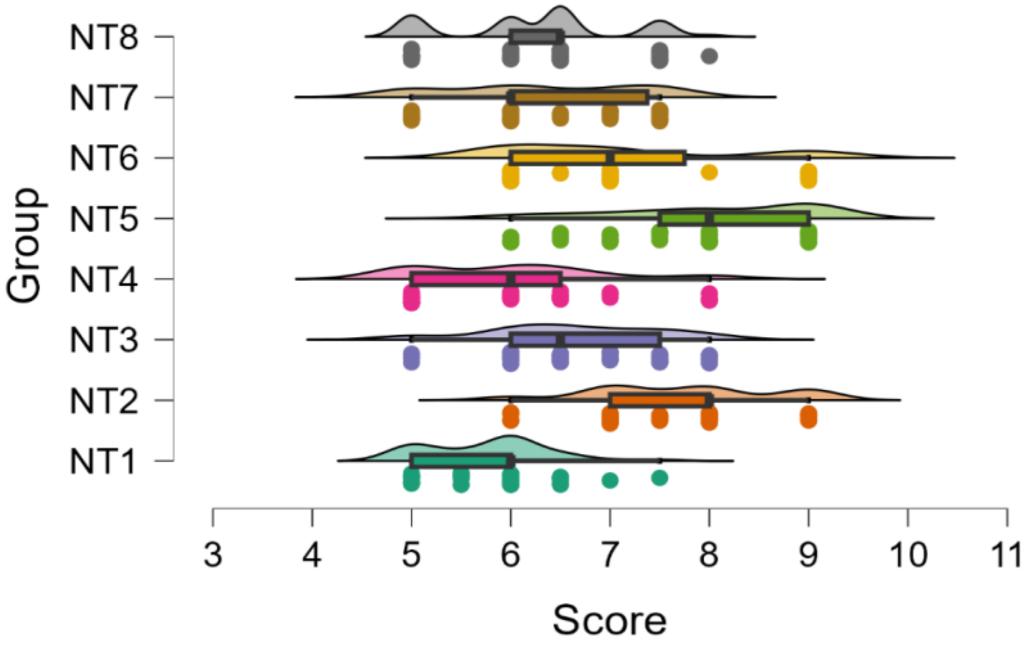

Figure 4. Data distribution collected using Raincloud (n=400).

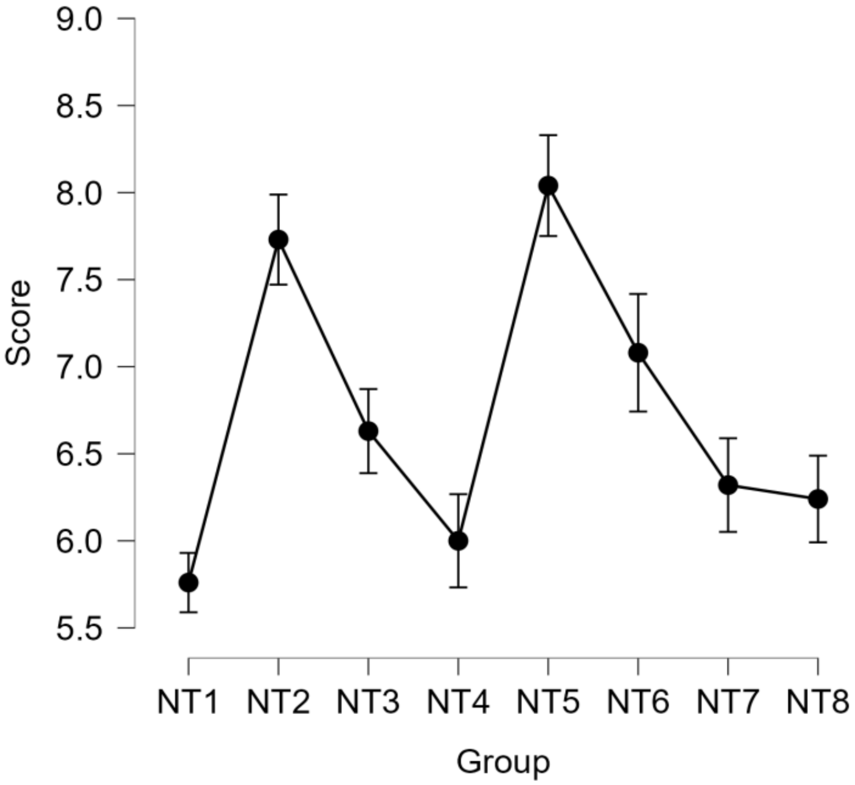

Figure 5. Average assessment scores of experiments (n=8).

To assess the homogeneity of means across different categories of a categorical variable, the null hypothesis (H0) is formally stated as: The population means of all categories are equal. The Brown-Forsythe and Welch tests were initially used to check this hypothesis, depending on whether the variances among the groups were equal or unequal. The results of the ANOVA test showed that the F-statistics from the Brown-Forsythe and Welch tests were significant ($p < 0.001$). Thus, there was a significant difference in the means of the 8 test groups (see Appendix 3).

Following the significant outcomes from the ANOVA analysis, we proceeded with the Tukey Honestly Significant Difference (HSD) test for post hoc analysis to pinpoint specific differences among the test formulations. Notably, formulations NT2 and NT5 stood out due to their elevated mean scores from consumer evaluations, indicating a pronounced preference when juxtaposed with other formulations. Figure 5 delineates that significant distinctions were found between NT2, NT5, and the remaining formulations, suggesting a distinct preference for NT2 and NT5 ($p < 0.05$), as detailed in appendix 4. Nonetheless, the comparison between NT2 and NT5 did not manifest a statistically significant variance, suggesting that while NT5 may be slightly preferred, the difference is not pronounced enough to be statistically validated. These insights are vital for selecting the optimal blend of formulations, taking into account the ingredient availability and cost efficiency, to maximize the product's market value.

CONCLUSION

The study of mixed composite products has demonstrated a highly reliable production process through testing that yielded negative results for harmful microorganisms on the ingredients in accordance with regulations. This approach can be applied to various component ingredients in the future when developing new mixing formulations. The sensory testing method involving product users was designed to be suitable in terms of the number of participants, compatibility, and standardization in data collection. The analysis results indicate changes in user preferences for mixed formulations, and these changes are statistically significant. However, at the two highest preference levels, there was insufficient evidence to conclude any significant difference. Scientists specializing in sensory evaluation or producers can use the results of this study to replace the two formulations with the highest preference that is suitable for the source materials and product costs, which has significant value for large-scale production processes.

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