Ingestion of Exopolymers from *Aureobasidium pullulans* Reduces the Duration of Cold and Flu Symptoms: A Randomized, Placebo-Controlled Intervention Study

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Aim. The objective of the study was to assess the efficacy of exopolymers from *Aureobasidium pullulans* (EAP) on the incidence of colds and flu in healthy adults. Methods. We conducted a randomized, double-blind, placebo-controlled study at the onset of the influenza season. A total of 76 subjects (30–70 years of age) were recruited from the general population. The subjects were instructed to take one capsule per day of either EAP or a placebo for a period of 8 weeks. The duration of cold and flu symptoms, a primary variable in assessing effectiveness, and serum cytokine levels as well as WBC counts as secondary variables were also evaluated. Results. EAP was associated with a statistically significant decrease in the duration of cold and flu symptoms, a primary variable in assessing effectiveness. Although cold and flu symptom levels were not significantly different at a significance level of 5%, the cold and flu symptom levels of the EAP group were less severe compared to the placebo group. No statistically significant changes of serum cytokine levels as well as WBC counts were observed. Conclusion. The results showed that EAP is a useful pharmaceutical and functional food material for preventing and treating colds and flu.

1. Introduction

The immune system has evolved to protect multicellular organisms, including humans, from pathogens. Immunostimulatory effects are regulatory actions that counteract reduced immune responses caused by immunodeficiency diseases, viral infections, malnutrition, tumors, and aging. Two strategies have been developed to stimulate the immune system. One method is to increase antigen-specific or non-specific immune responses, and the other method involves the use of appropriate immunosupplements for antigen administration [1].

Respiratory viruses are among the most infectious pathogens in humans, and many differences occur in the kinds of pathogens, their antigenicities, and their worldwide infection patterns. Recently, respiratory diseases such as Middle East respiratory syndrome, severe acute respiratory syndrome, and the flu caused by novel swine-origin influenza A strains have emerged and begun to spread rapidly [2–4]. As the associated respiratory viruses show very strong tendencies to spread, there is a growing interest in foods that promote immune functions as well as a global monitoring system for influenza [5].

The results of several researches have revealed a positive correlation between food nutrition and the control of human immune functions, and various foods have been recognized as functional foods that can improve immune functions. These food materials may be derived from vegetables [6],...
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**Figure 1:** Schematic representation of this clinical study. ITT, intention to treat; PP, per protocol.

surgery (excluding a simple appendectomy or hernia operation); individuals who consumed medicine or herbal medicines within a month of participation in the experiment which could affect immunity; individuals who participated in a different human study or clinical test and took experimental products within 3 months of participation in this experiment (excluding human studies with cosmetics); and individuals whom the researchers otherwise determined might have difficulty completing the experiment.

2.2.3. **Withdrawal.** Dosages of the experimental products were temporarily suspended at instances where it was appropriate to stop the experiment for the welfare of the subjects.

2.2.4. **Temporary Suspension for the Treatment of Adverse Events That May Have Occurred.** In the following cases, administration of the test foods was temporarily suspended: (a) a temporary interruption on the occurrence of an adverse reaction or for the treatment of an adverse reaction; (b) when showing adverse events related to the safety of the test foods; and (c) in case of showing acute reactions (allergies and hypersensitivity) to the test foods. However, in the case of a temporary interruption for the occurrence of an adverse reaction or for the treatment of an adverse reaction, if there was no problem in its compliance, the study was continued.

2.2.5. **Dropout.** Cases that were processed as dropouts included those in which test foods were allocated and the subject or a legal representative withdrew consent from participation in the experiment, cases where results for a final inspection could not be obtained, cases where subjects did not adhere to the test protocol resulting in a significant effect on the test results, cases where there was an interruption in contact with the subject (i.e., when the subject could not be traced), cases where the subject failed to make visits, subjects for whom a disease was discovered which went unnoticed during the screening inspection, cases where compliance with usage of the test food fell below 80%, as assessed with the usage log, cases in which, without the direction of the researcher, the subject used a medicine or product while they were taking the test food which could affect the research results or interpretation of data, cases in which the subject had an average weekly drinking quantity as ethanol of 490 g/week throughout the test period, or cases where the lead researcher determined that the research proceedings were inappropriate or that there could be a significant effect on the safety of the subjects or the experiment results.

Subjects stopped taking test foods and were processed as dropouts for the following reasons, in which it was not possible to continue the human study: cases in which a serious adverse event occurred with the subject, cases in which an adverse event made participation in the experiment impossible, cases in which the researcher determined that the usage and observation of the test foods would be impertinent, or cases in which clinical tests were not possible due to the death of a subject or emergence of a disease.

Subjects who took the test foods and later dropped out of the study, where a portion or the entire estimate value of the final assessment (visit 3) was omitted, were included in the ITT group’s analysis data. Missing values were processed after a dropout occurred using missing value processing method for effective assessments. The results of all subjects who dropped out were excluded from assessment of the per protocol (PP) group (Figure 1 and Table 1).

2.2.6. **Drinking, Diet, and Exercise among the Subjects.** During the experiment, subjects maintained regular levels of drinking, diet, and exercise which were similar to levels prior to participation in the experiment. To verify this, on the day of base evaluations, we examined the dietary contents
2.4. Basic Information and Vital Signs of Subjects

2.4.1. Visitation Schedule. The visitation dates allowed for the set base visitation days + 5 additional days. Those who were selected as test subjects received the test foods within 14 days from the base evaluation date (selection inspection date) and began taking the test foods by the next morning.

2.4.2. Basic Information of Subjects. Demographic data including the initials, age, gender, birthdate, weight, and height of the subjects and basic information such as medical history, medicinal intake history, combined treatment, concomitant drugs, smoking, drinking, and exercise habits were recorded in detail. The contents during the last 3 years were recorded in the medical histories, and contents during the last 4 weeks were recorded in the medicinal intake history. The types of exercise and the exercise times were examined for 1 week prior to the time of the visitation day. Diet and exercise were compared during the base evaluation period and experimental period and observed to determine whether any important changes occurred which could affect the test results. The number and quantity of alcoholic drinks consumed during the week preceding the visitation day were examined, and that information was converted into the number of grams of ethanol consumed and recorded.

2.4.3. Vital Signs and Obesity Levels. Blood pressure, body temperature, and pulse were measured and recorded as vital signs. Obesity levels were measured using a body fat analyzer (Inbody 3.0, Biospace, Korea), and the BMI (kg/m²), percent...
Table 2: Symptom level of illness, such as cold and flu.

| Symptom level | Score | Symptom levels reported by the patients |
|---------------|-------|----------------------------------------|
| No symptom    | 0     | I did not perceive relevant symptoms    |
| Mild          | 1     | I perceived some symptoms, but they did not interfere with my daily life |
| Moderate      | 2     | I perceived symptoms and felt discomfort in my daily life. I was able to take general pharmaceuticals without causing an issue in my daily life |
| Severe        | 3     | My daily life was very uncomfortable due to symptoms, and proactive treatment was needed. In the case of severe discomfort, I may stop working or studying or be admitted to the hospital |

body fat (%), waist-hip ratio, and visceral fat area (cm²) were recorded.

2.5. Test Items. Hematological examination of WBCs, red blood cells, hemoglobin levels, hematocrits, and platelets was performed using an automated analyzer (Sysmex XP-300; Sysmex, Kobe, Japan). The levels of blood urea nitrogen, creatinine, uric acid, total bilirubin, albumin, total protein, alkaline phosphatase, glucose, alanine transaminase, aspartate transaminase, gamma-glutamyl transferase, triglyceride, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol were measured using a clinical chemistry analyzer (Dimension Xpand Plus; SIEMENS, Munich, Germany). All urine samples were stored in the dark at 4°C and were analyzed within two hours of collection. The pH, specific gravity, and protein and glucose levels were measured using a semiautomated analyzer (URiSCAN Pro II; YD Diagnostics, Seoul, Korea). Pregnancy testing was performed using a kit detecting human chorionic gonadotropin. Serum concentration of cytokines was analyzed by using hMAGLXsa (6 PLEX) Multiplex ELISA kit (R&D Systems, Minneapolis, MN). Cytokine levels were determined using LumineX 200 (LumineX, Austin, TX, USA) and the data were reported as median fluorescent intensities.

2.6. Preliminary/Postsurvey Comparisons. If immune functions improve, the disease duration and symptom levels should decrease due to increased resistance to viral diseases such as colds and flu. Accordingly, we examined the duration and levels of cold and flu symptoms after consuming the test food products as a functional index of immunological improvements. For the survey following the administration of test foods, a form was provided in which subjects made a daily record of their health conditions, with a daily record of their test food dosages. Nine symptoms were monitored to inspect the health conditions of the subjects in the survey: fever, chills, runny nose, nasal congestion, coughing, sneezing, sore throat, headache, and gastrointestinal symptoms (nausea, vomiting, diarrhea, and abdominal pain). Subjects marked O for “symptoms exist” and × for “no symptoms.” To record the symptom levels, subjects used a 4-step scoring point system, where they marked (0) for no symptoms, (1) for minor symptoms, (2) for moderate symptoms, and (3) for serious symptoms [27] and marked whether they took early leave or were absent from work or school due to symptoms. Table 2 shows a detailed explanation of the criteria used for reporting symptom levels. To assess the effectiveness of the experimental food products, subjects kept daily records in their dosage logs of cold and flu symptom durations and symptom levels during the testing period.

2.7. Compliance Evaluation. Compliance with ingesting test foods was evaluated based on the food-usage logs. During the final visit, the number of remaining foods collected was compared with the usage log records. In cases where there were differences between the recorded and actual amounts of food taken, compliance was verified when the differences were small.

2.8. Effectiveness Assessment Variables. Primary effectiveness assessment variables included the duration and total score of symptom levels for cold and influenza. Secondary effectiveness assessment variables included serum IL-1β, IL-6, IL-8, IL-10, TNF-α, and INF-γ levels, as well as WBC counts and differential blood counts.

2.9. Safety Assessment

2.9.1. Groups Evaluated. The targets of evaluation included subjects who had taken (even once) foods used in the human study and who had been visited at least one time, from whom safety data in terms of adverse events, vital sign values, or changes in clinical examination values were procured.

2.9.2. Evaluation Parameters. Subjects were evaluated in terms of predicted side effects, adverse events and adverse drug reactions, abnormal changes in clinical examination values, and vital signs.

2.10. Evaluation Methodology. A comparison was made of the side effects, adverse drug reactions, and the manifestation and frequency of adverse events, with a cause-and-effect relationship with the test foods predicted to occur in the EAP and placebo groups. Differences between the groups were analyzed by performing a chi-squared test and Fisher’s exact test.

2.11. Statistical Analysis. Statistical analysis for assessing the effectiveness of test foods was based on the Statistical Guidelines for Clinical Trials [28], and the statistical package used was the SAS (version 9.4) on Microsoft Windows. The statistical significance level was set at 5%. In other words, there was statistical significance if the p value (significance probability) was below 0.05. Statistical analysis was performed by focusing
on significance comparisons between the EAP and placebo groups. The methodologies described below were followed for detailed analysis.

2.12. Analysis Sets. Analysis sets obtained from the test subjects were composed largely of an ITT analysis group and a PP analysis group. The ITT group comprised the primary data set used for effectiveness assessments, and the PP group was additionally analyzed to assess the effectiveness. Subjects in the ITT group included those who had taken the test food materials and whose assessment variables were measured at least once during visit 1. Subjects in the PP group included those in the ITT group who completed the test according to the study protocol.

2.13. Statistical Analysis Items

2.13.1. Baseline Observation Items Prior to Food Ingestion. Items for a baseline homogeneity test between the test groups are gender, age, weight, height, obesity, alcoholic drink consumption, exercise, and smoking.

2.13.2. Pre/Post Food Ingestion Observation Items. Items for a pre/post food ingestion homogeneity test within the test groups are weight, height, alcoholic drink ingestion, and exercise. Items for an effectiveness test are duration and symptom levels of cold and flu symptoms; serum IL-1β, IL-6, IL-8, IL-10, TNF-α, and INF-γ levels; and WBC and differential blood counts. Other observation items are clinical laboratory test items.

2.13.3. Homogeneity Test. A homogeneity test was carried out between the EAP and placebo groups on the baseline observation items prior to the ingestion of test foods. In this study, chi-square method was conducted for category variables or Fisher's exact test was performed if over 25% of the cells had an expected frequency of less than 5. In the homogeneity test for continuous variables, independent t-test was conducted. In addition, analytical methods were employed using the above homogeneity tests to investigate whether weight, obesity, alcoholic drink ingestion, or exercise could have affected the test results, due to changes in lifestyle habits during the test period.

2.13.4. Effect Test. It has been predicted that fewer subjects will contract viral diseases such as cold and flu if their immune functions are enhanced and that the duration and levels of symptoms will decrease if one of the diseases is contracted. The activation state of principal cells related to immunity can be determined by measuring changes in the serum contents of cytokine IL-1β, IL-6, IL-8, IL-10, TNF-α, and INF-γ. To assess the effectiveness of the test food materials, the significance of the mean difference (visits 1–3) was tested for each variable included in the effectiveness test. In other words, a statistical comparison was made between the average variation before and after food ingestions in the EAP and placebo groups. To this end, normality was tested for the evaluation data. The parametric method of a two-sample t-test was chosen if normality was satisfied, whereas the nonparametric method of a Wilcoxon's rank-sum test was chosen if normality was not satisfied. In addition, the above significance tests were performed on mean differences between the EAP and placebo groups between each visit after test food ingestions. To analyze the prevalence of cold symptoms, subjects recorded the duration and levels of symptoms when they contracted a cold or the flu prior to ingesting the test food. During visit 3, subjects recorded the duration and total symptom scores of cold and influenza symptoms during the test period. Accordingly, it was not meaningful to compare the mean difference between visits, considering that the rating scales used in visits 1 and 3 were different. In other words, we confirmed homogeneity between the test groups in visit 1 and evaluated the effectiveness by comparing the results between test groups in visit 3.

2.13.5. Analysis of Missing Values. For the ITT group, an analysis is often conducted with the Last Observation Carried Forward (LOCF) method (a single-imputation method) for data missing in the final effectiveness assessment. Typically, the LOCF method is easy to use in clinical trials, despite the restricting assumption where missing data are treated as a constant. The LOCF method is commonly used because it provides conservative results. However, there are no commonly recommended methodologies for processing missing values [29]. In this study, the statistical analytic data collected was comprised of only the baseline assessment prior to the ingestion of test food materials and the final assessment after ingestion of test food materials. The missing values during the final assessment were not replaced by the base assessment values but were substituted by the average of each group and assessed.

3. Results

3.1. Subjects Participating in This Study. In this study, we screened 117 individuals who voluntarily agreed to participate in this human study, and 76 subjects who met the selection criteria and did not meet any exclusionary criteria were enrolled. Among the 76 subjects, 38 were assigned to the EAP group and 38 were assigned to the placebo group (Figure 1).

3.2. Violations of the Study Protocol. Four instances were verified in which subjects violated the study protocol while consuming the test foods. These 4 subjects were processed as dropouts.

3.3. Dropouts. Among the 76 subjects who were selected, 2 individuals from the EAP group dropped out due to violations in the visitation schedule and shortfalls in compliance, and 2 individuals from the placebo group dropped out due to withdrawal of informed consent and shortfalls in compliance. The remaining 72 subjects adhered to the test protocol and completed the test. The current states of dropouts are separated according to the test group (Figure 1).
Table 3: Foundational statistics and homogeneity testing for compliance with ingesting test foods, subject age, number of days consuming alcoholic drinks, and quantity of alcoholic drinks.

| Variable               | EAP group | Placebo group | t-value | p value |
|------------------------|-----------|---------------|---------|---------|
|                        | Mean      | Std. dev.     | Mean    | Std. dev. |       |       |
| Compliance             | 93.82     | 6.73          | 94.81   | 6.09     | 0.66  | 0.5140 |
| Age                    | 46.42     | 10.09         | 48.86   | 9.89     | 1.04  | 0.3026 |
| Drinking               |           |               |         |          |       |       |
| Visit 1                |           |               |         |          |       |       |
| No. of days drinking   | 1.78      | 1.06          | 2.00    | 1.00     | 0.64  | 0.5285 |
| Amount of drinks consumed (g alcohol) | 68.36 | 54.28         | 70.41   | 84.55    | 0.09  | 0.9321 |
| Visit 3                |           |               |         |          |       |       |
| Total no. of days drinking | 9.74  | 5.72          | 10.05   | 8.38     | 0.14  | 0.8928 |
| Amount of drinks consumed (g alcohol) | 415.50 | 350.80        | 407.70  | 402.60   | -0.07 | 0.9484 |

Table 4: Foundational statistics and homogeneity testing of subject gender, drinking, exercise, smoking, medical history, and medications.

| Variable               | Group | Male Frequency | % | Female Frequency | % | Chi-squared value | p value |
|------------------------|-------|----------------|---|------------------|---|-------------------|--------|
| Gender                 | EAP group | 7 | 19.44 | 29 | 80.56 | 2.4923 | 0.1144 |
|                        | Placebo group | 13 | 36.11 | 23 | 63.89 |
| Drinking               | EAP group | 18 | 50.00 | 18 | 50.00 | 0.0556 | 0.8136 |
|                        | Placebo group | 17 | 47.22 | 19 | 52.78 |
| Smoking                | EAP group | 28 | 77.78 | 8 | 22.22 | 0.0000 | 1.0000 |
|                        | Placebo group | 28 | 77.78 | 8 | 22.22 |
| Medical history        | EAP group | 2 | 5.56 | 34 | 94.44 | 2.0571 | 0.1515 |
|                        | Placebo group | 0 | 0.00 | 36 | 100.00 |
| Medications            | EAP group | 1 | 2.78 | 35 | 97.22 | 1.9343 | 0.1643 |
|                        | Placebo group | 4 | 11.11 | 32 | 88.89 |
| Concomitant drug admin. | EAP group | 3 | 8.33 | 33 | 91.67 | 0.0000 | 1.0000 |
|                        | Placebo group | 3 | 8.33 | 33 | 91.67 |

3.4. Compliance Assessment. Compliance with ingesting test food materials was assessed in visit 3. The contents recorded in the daily record filled out by the subjects as well as the quantity of remaining products returned during the final visit were confirmed and assessed. Compliance was calculated using the following formula: compliance (%) = (number of food materials ingested/number of food materials that should have been ingested) × 100, where the number of food materials ingested = number of food materials provided − number of food materials returned.

Subjects whose compliance was below 80% were set as dropouts, and there were 2 dropouts in this study due to compliance shortfalls. The compliance of test food material ingestion was 93.82 ± 6.73% in the EAP group and 94.81 ± 6.09% in the placebo group. A significant difference in compliance between the test groups was not observed (Table 3).

3.5. Homogeneity Test. A homogeneity test was conducted between test groups for the following: measurement values prior to food material ingestion regarding demographic information of the subjects including age, gender, medical history, treatment history, medication history, and concomitant medication and measurement values before and after ingestion of food materials regarding items that could affect the test results based on changes in lifestyle habits during the test period including regular exercise, smoking, and drinking. The results of a preliminary investigation on the age, gender, medical history, treatment history, medication history, concomitant medication, exercise, smoking, and drinking among the subjects are summarized in Tables 3 and 4. No significant differences were found between the test groups regarding any of the items examined.
Table 5: Foundational statistics and comparisons of blood test results between test groups.

| Variable       | Group          | Mean   | Std. dev. | T-value | p value |
|----------------|----------------|--------|-----------|---------|---------|
| White blood cell | EAP group      | -0.41  | 1.07      | 0.31    | 0.6225  |
|                | Placebo group  | -0.32  | 1.25      |         |         |
| Neutrophil     | EAP group      | -0.079 | 8.07      | -0.39   | 0.3489  |
|                | Placebo group  | -0.81  | 8.29      |         |         |
| Eosinophil     | EAP group      | -0.04  | 1.19      | -0.39   | 0.4717  |
|                | Placebo group  | -0.06  | 0.93      |         |         |
| Basophil       | EAP group      | -0.05  | 0.27      | -0.82   | 0.2086  |
|                | Placebo group  | -0.10  | 0.26      |         |         |
| Lymphocyte     | EAP group      | -0.38  | 6.90      | 0.68    | 0.7491  |
|                | Placebo group  | 0.70   | 7.10      |         |         |
| Monocyte       | EAP group      | 0.55   | 2.25      | -0.53   | 0.2973  |
|                | Placebo group  | 0.2676 | 2.40      |         |         |

Missing values were replaced with averages and used in the ITT group data analysis.

3.6. Analyzing the Results of Primary Effectiveness Assessment Variables in the ITT Group. The results of a t-test performed to evaluate significant differences between 2 groups before and after ingesting the test food materials verified that the duration of cold symptoms (p value = 0.5748) and cold symptom levels (p value = 0.2462) at visit 1 were not significant at a significance level of 5%. Statistical analysis also showed that no difference occurred between test groups prior to the ingestion of food materials. During visit 3, the duration of cold symptoms in the experiment group (3.1579 ± 4.4814 days) was lower than that of the placebo group (6.6842 ± 9.3581 days). The difference between the test groups was statistically significant (p value = 0.0410). The EAP group also had a lower score for cold symptom levels than the placebo group (8.7105 ± 13.7269 versus 18.3421 ± 30.1566, resp.). This difference was significant at a level of 10% (p value = 0.0790) but not 5% (Figure 2).

3.7. Analysis of Secondary Effectiveness Assessment Variables in the ITT Group

3.7.1. Blood Test Results. Significant differences (visits 1–3) were verified with respect to the mean difference of each measured item between the visitation times to assess the effectiveness of the test food materials on the blood test results. The results were not significantly different between the EAP and placebo groups (Table 5) at a significance level of 5%. For WBC, basophil, and lymphocyte counts that
Table 6: Comparison (nonparametric statistical analysis) of the median of the subjects’ blood test results.

| Variable       | Group       | Expected value under H0 | Std. dev. under H0 | Mean score | Z-value | p value |
|----------------|-------------|--------------------------|--------------------|------------|---------|---------|
| White blood cell | EAP group   | 1,463                    | 96.21              | 37.00      | -0.5872 | 0.2785  |
|                | Placebo group | 1,463                  | 96.21              | 40.00      |         |         |
| Basophil       | EAP group   | 1,463                    | 95.83              | 40.13      | 0.6418  | 0.2605  |
|                | Placebo group | 1,463                  | 95.83              | 36.87      |         |         |
| Lymphocyte     | EAP group   | 1,463                    | 96.26              | 37.41      | -0.4259 | 0.3351  |
|                | Placebo group | 1,463                  | 96.26              | 39.60      |         |         |

Wilcoxon’s rank-sum test was used to analyze variables that did not follow normality.

Table 7: Foundational statistics and comparison between test groups in terms of serum cytokine levels.

| Variable | Group       | Mean   | Std. dev. | T-value | p value |
|----------|-------------|--------|-----------|---------|---------|
| IFN-γ    | EAP group   | -1.12  | 6.57      | 0.36    | 0.6398  |
|          | Placebo group | -0.51  | 8.07      |         |         |
| IL-1β    | EAP group   | 0.52   | 2.28      | -0.26   | 0.3987  |
|          | Placebo group | 0.40   | 1.96      |         |         |
| IL-10    | EAP group   | 0.31   | 1.29      | -0.81   | 0.2107  |
|          | Placebo group | -0.24  | 4.01      |         |         |
| IL-6     | EAP group   | 0.21   | 0.78      | -0.93   | 0.1782  |
|          | Placebo group | 0.01   | 1.07      |         |         |
| IL-8     | EAP group   | -10.53 | 47.64     | 1.18    | 0.8772  |
|          | Placebo group | -1.36  | 5.00      |         |         |
| TNF-α    | EAP group   | 0.96   | 1.41      | -0.69   | 0.2464  |
|          | Placebo group | 0.74   | 1.31      |         |         |

Missing values were replaced with averages and used in the ITT group data analysis.

Table 8: Comparison (nonparametric statistical analysis) of serum cytokine levels.

| Variable | Group       | Expected value under H0 | Std. dev. under H0 | Mean score | Z-value | p value |
|----------|-------------|--------------------------|--------------------|------------|---------|---------|
| IL-10    | EAP group   | 1,463                    | 96.26              | 40.43      | 0.7584  | 0.2241  |
|          | Placebo group | 1,463                  | 96.26              | 36.57      |         |         |
| IL-6     | EAP group   | 1,463                    | 96.24              | 39.86      | 0.5299  | 0.2981  |
|          | Placebo group | 1,463                  | 96.24              | 37.14      |         |         |
| IL-8     | EAP group   | 1,463                    | 96.26              | 36.58      | -0.7532 | 0.2257  |
|          | Placebo group | 1,463                  | 96.26              | 40.42      |         |         |
| TNF-α    | EAP group   | 1,463                    | 96.20              | 39.64      | 0.447   | 0.3275  |
|          | Placebo group | 1,463                  | 96.20              | 37.36      |         |         |

A Wilcoxon’s rank-sum test was used for variables that do not follow normality.

3.7.2. Cytokine Test Results. To assess the effectiveness of the test food materials, a t-test was conducted to evaluate significance differences in the mean cytokine concentrations between visits 1–3. The results were not significantly different between the EAP and placebo groups (Table 7) at a significance level of 5%.

3.8. Safety Evaluation Results

3.8.1. Adverse Events. Adverse events were observed in 3 individuals from the placebo group and in 1 individual from the EAP group. In the placebo group, nausea and pruritus were confirmed in subject E11, asthenia and rash in subject E49, and a sore throat in subject E68. No other adverse events were observed aside from these. In the EAP group, headache was confirmed in subject E59, but no other adverse events were confirmed aside from this. All these adverse events subsided within a few days. In the case of subject E59, who ingested the test food materials, the headache was completely alleviated after 2 days, and the subject was judged as having no cause-and-effect relationship with ingesting the test food materials (data not shown).
3.8.3. Overall Safety Level. With the occurrence of adverse events and abnormal changes in clinical test results, no items were found to correlate with the use of test food materials or were suspected to have a correlation. Thus, the test foods were found to be safe in the context of this study.

4. Discussion

4.1. Effects of EAP against Colds and Flu. The various biological activities of β-glucan were verified through in vitro testing, animal testing, and clinical trials [12]. Recently, the effectiveness of β-glucan on respiratory diseases has been studied in rodent [30] and human [31–33] because their prevalence has increased rapidly. EAP, exopolymers purified from Aureobasidium pullulans SM-2001, containing 13%β-1,3/1,6-glucan [20], are used in test foods and have shown potent immunomodulatory activities in a mouse model [16]. However, this study is the first to evaluate the use of EAP against respiratory diseases.

To assess the ability of EAP to improve human immunological responses and to alleviate cold or flu symptoms, 117 adults aged 30–70 years with a white blood cell count of 4–7 x 10³/µl were screened. 76 individuals were enrolled as subjects who satisfied the selection criteria and did not fall under any exclusion criteria (data not shown). In an aspect of subject’s respiratory morbidity, none of the subjects had a respiratory disease at the time of enrollment. Within 4 weeks prior to the screening date of the subjects, there were 2 subjects with light respiratory disease. After confirming no symptoms at the enrollment, the 2 subjects were proceeded to obtain informed consent.

The 76 subjects (administered with the test foods for a period of 8 weeks) were randomly assigned to either EAP or a placebo group. Blood tests and surveys of cold or flu symptoms were conducted before and after ingesting test food materials, and the duration and symptom level of colds or flu, as well as the results of the blood test and cytokine analysis, were compared. The EAP and placebo groups were composed of individuals that exhibited homogeneity and showed no statistically significant differences in their compliance of ingesting test foods or in gender, age, compliance, regular exercise, smoking, drinking, medical history, medicinal history, or concomitant drug administration.

Clinical data have shown that immunity-promoting functions can prevent acute respiratory infections from cold or influenza viruses and reduce the frequency of contracting colds or flu, symptom levels, and symptom durations based on effectiveness criteria [34]. In this study, statistical analysis of the duration and level of cold symptoms, a primary variable for assessing effectiveness, showed that there were no differences in assessment values between the EAP and placebo groups before ingesting test food materials.

However, the duration of cold and flu symptoms after ingesting test food materials was significantly lower in the EAP group than in the placebo group at a 5% significance level. The EAP group also showed lower levels of cold and flu symptoms, which were statistically different at the 10% (but not 5%) significance level than placebo group. It is predicted that individuals whose immune functions are enhanced will contract fewer viral diseases such as colds and flu, with reduced durations and levels of symptoms. The activation state of primary cells related to immunity can be determined by measuring the serum levels of the cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α, and INF-γ [1]. According to the "Guideline for Safety and Efficacy Assessments of Functional Food on Immune Function Improvement" from the Ministry of Food and Drug Safety of Korea, changes in cytokine levels, WBC counts, T cell counts, and NK cell activity in the peripheral blood should be measured when determining the functionality of immunity reinforcement in humans [35].

In this study, secondary variables including serum cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α, and INF-γ) and WBCs (neutrophil, eosinophil, basophil, lymphocyte, and monocyte) for assessing effectiveness did not exhibit statistically significant differences before and after ingestion of test food materials at a significance level of 5%. These results are consistent with those of Choi et al. (2009) [14], where the daily intake level of EAP (Polycan™) or placebo food materials was set at 400 mg, and changes in serum cytokines were studied (with the intention of observing effectiveness
in immunity improvements of EAP), but no statistically significant changes were found.

Although EAP has shown potent immunomodulatory activities in a mouse model [16], no immunity improvement of EAP on the human was observed in this study. The cytokine is a factor that needs to change suddenly according to the individual health condition and then return to the normal range [1]. On the normal range of blood cytokine concentration, its individual variation is very large. Thus, a large number of subjects are required to measure the normal range of cytokine concentrations [36]. Indeed, in a study of normal Korean cytokine levels, 110 subjects were studied [37], and in a study of normal American cytokine levels, 144 subjects were examined [38] to estimate the normal range of blood cytokine concentration. Several studies have shown that no changes in blood cytokine levels have been observed following ingestion of multivitamin and mineral supplement [39], probiotic supplement [40], fish oil [41], and Cordyceps militaris [42], which are known to help improve immunity. This may be because the cytokine level increased during the test period and then returned to normal, or the number of subjects was small, indicating that the individual deviation range was not statistically significant. In this study, the total number of subjects was 72: 36 in the test group and 36 in the control group, respectively. Therefore, the results of this study may not statistically overcome the individual variation range of the subject’s cytokine level due to the small number of subjects. Further research is needed on extensive clinical studies with clear statistical numberings to confirm the effects of EAP on plasma cytokines.

No significant adverse events occurred during the period of test food material intake, and adverse events (excluding colds) were observed in 3 individuals from the placebo group (nausea, pruritus, asthenopia, rash, and sore throat) and in 1 individual from the experiment group (headache). All these adverse events subsided within just a few days and were determined to have no cause-and-effect relationship with the ingestion of test food materials. Changes in blood test values were also observed in the EAP group with an increase in SGPT, TG, and γ-GTP values, but this was difficult to determine as being clinically abnormal. In addition, in an aspect of the respiratory morbidity, the subjects who suffered from cold during the test period appeared and then fully cured after the treatment, and no respiratory morbidity was shown in all subjects. Therefore, EAP was verified to be harmless to the human body.

4.2. Conclusion. Collectively, our data revealed that EAP was associated with a statistically significant decrease in the duration of cold and flu symptoms, a primary variable in assessing effectiveness. Although cold symptom levels were not different at a significance level of 5%, the cold and flu symptom levels of the EAP group were less severe compared to the placebo group. No statistically significant changes were observed in the serum cytokine concentrations, WBC, and differential blood counts (secondary variables for assessing effectiveness). Therefore, our data showed that EAP is a useful medicine and functional food material for preventing and treating colds and flu.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors declare that there are no conflicts of interest.

Authors’ Contributions
Jong-Min Lim and Eunju Do contributed equally to this work.

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References
[1] J. A. Owen, J. Punt, and S. A. Stranford, KUBY Immunology, Freeman and Company, New York, NY, USA, 7th edition, 2013.
[2] Z. A. Memish, M. Cotten, B. Meyer et al., “Human Infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013,” Emerging Infectious Diseases, vol. 20, no. 6, pp. 1012–1015, 2014.
[3] A. Y. Y. Wong, H. Chen, S.-H. Liu et al., “From SARS to avian influenza preparedness in Hong Kong,” Clinical Infectious Diseases, vol. 64, pp. 598–610, 2017.
[4] P. C. Resende, P. S. Born, A. R. Matos et al., “Whole-genome characterization of a novel human influenza A(HIN2) virus variant, Brazil,” Emerging Infectious Diseases, vol. 23, no. 1, pp. 152–154, 2017.
[5] H. Ham, J. Jang, S. Choi et al., “Epidemiological Characterization of Respiratory Viruses Detected from Acute Respiratory Patients in Seoul,” Annals of Clinical Microbiology and Antimicrobials, vol. 16, no. 4, p. 178, 2013.
[6] S. Kumar, P. Gupta, S. Sharma, and D. Kumar, “A review on immunostimulatory plants,” Journal of Chinese Integrative Medicine, vol. 9, no. 2, pp. 117–128, 2011.
[7] Y. S.-K. Kim and E. Mendis, “Bioactive compounds from marine processing byproducts—a review,” Food Research International, vol. 39, no. 1, pp. 383–393, 2006.
[8] H. A. El Enshasy and R. Hatti-Kaul, “Mushroom immunomodulators: unique molecules with unlimited applications,” Trends in Biotechnology, vol. 31, no. 12, pp. 668–677, 2013.
[9] L. K. Erickson and N. E. Hubbard, “Probiotic Immunomodulation in Health and Disease,” Journal of Nutrition, vol. 130, no. 2, pp. 403S–409S, 2010.
[10] Y. Kato, H. Onishi, and Y. Machida, “Application of chitin and chitosan derivatives in the pharmaceutical field,” Current Pharmaceutical Biotechnology, vol. 4, no. 5, pp. 303–309, 2003.
[11] H. Korhonen and A. Pihlanto, “Bioactive peptides: production and functionality,” International Dairy Journal, vol. 16, no. 9, pp. 945–960, 2006.
[12] K. M. I. Bashir and J.-S. Choi, “Clinical and physiological perspectives of β-glucans: the past, present and future,” International Journal of Molecular Sciences, vol. 18, no. 1906, pp. 1–46, 2017.
[13] S. O. Park and J. M. Kim, “Functional food for immune regulation- Beta-glucan,” Korean Journal of Food Science and Technology, vol. 45, no. 1, pp. 39–47, 2012.

[14] H. Y. Choi, J. D. Kim, and M. Y. Park, “A 4 week randomized, double-blind human trial to compare the efficacy and safety of Aureobasidium pullulans cultured solution and placebo on improvement of immune in subjects,” Korean Journal of Medicine, vol. 15, no. 3, pp. 83–91, 2009.

[15] A. Estrada, C.-H. Yun, A. Van Kessel, B. Li, S. Haua, and B. Laarveld, “Immunomodulatory activities of oat β-glucan in vitro and in vivo,” Microbiology and Immunology, vol. 41, no. 12, pp. 991–998, 1997.

[16] H. S. Yoon, J. W. Kim, H. R. Cho et al., “Immunomodulatory effects of Aureobasidium pullulans SM-2001 exopolymers on the cyclophosphamide-treated mice,” Journal of Microbiology and Biotechnology, vol. 20, no. 2, pp. 438–445, 2010.

[17] E. Lotzova and J. U. Gutterman, “Effect of glucan on natural killer (NK) cells: Further comparison between NK cell and bone marrow effector cell activities,” The Journal of Immunology, vol. 123, no. 2, pp. 607–611, 1979.

[18] J.-N. Lee, D.-Y. Lee, I.-H. Ji et al., “Purification of soluble β-glucan with immune-enhancing activity from the cell wall of yeast,” Bioscience, Biotechnology, and Biochemistry, vol. 65, no. 4, pp. 837–841, 2001.

[19] J. L. Benach, G. S. Habicht, T. W. Holbrook, and J. A. Cook, “Glucan as an adjuvant for a murine Babesia microti immunization trial,” Infection and Immunity, vol. 35, no. 3, pp. 947–951, 1982.

[20] K. Hirabayashi, N. Kondo, and S. Hayashi, “Characterization and enzymatic hydrolysis of hydrothermally treated β-1,3–1,6-glucan from Aureobasidium pullulans,” World Journal of Microbiology and Biotechnology, vol. 32, no. 12, article no. 206, 2016.

[21] P. Lotrakul, P. Unhapattaratitikul, T. Seelanan, S. Prasongsuk, and H. Pannapayak, “An aubasidan-like β-glucan produced by Aureobasidium pullulans in Thailand,” ScienceAsia, vol. 39, no. 4, pp. 363–368, 2013.

[22] N. Hamada and Y. Tsujioka, “The Structure of the Carbohydrate Moiety of an Acidic Polysaccharide Produced by Aureobasidium sp. K-4,” Agricultural and Biological Chemistry, vol. 47, no. 6, pp. 1167–1172, 1983.

[23] H. P. Seo, J. M. Kim, H. D. Shin et al., “Production of 6l, 3l, 6glucan by Aureobasidium pullulansSM2001,” Korean Journal of Biotechnology and Bioengineering, vol. 17, no. 4, Article ID 376380, 2002.

[24] J.-S. Choi, M.-Y. Park, J.-D. Kim, H. R. Cho, I. S. Choi, and J.-W. Kim, “Safet y and efficacy of polycalcium for improving biomarkers of bone metabolism: A 4-week open-label clinical study,” Journal of Medicinal Food, vol. 16, no. 3, pp. 263–267, 2013.

[25] J. D. Kim, M. Y. Park, J. W. Kim et al., “Randomized, double-blind, placebo-controlled trial of the effects of Polycan, β-glucan originating from Aureobasidium pullulans, on bone biomarkers in healthy women,” Journal of Physiology Pathology in Korean Medicine, vol. 29, no. 4, pp. 330–336, 2015, In Korean with English abstract.

[26] H.-S. Park, M.-S. Kang, Y.-M. Kim et al., “A clinical study for the efficacy and safety of β-glucan from Aureobasidium pullulans in mild to moderate atopic dermatitis patients,” International Journal of Molecular Medicine, 2018, in press.

[27] K.-C. Ha, M.-G. Kim, M.-R. Oh et al., “A placebo-controlled trial of Korean red ginseng extract for preventing Influenza-like illness in healthy adults,” BMC Complementary and Alternative Medicine, vol. 12, article no. 10, 2012.

[28] Korea Food and Drug Administration (KFDA), Guidelines for clinical trial statistics, KFDA, Seoul, Korea, 2000.

[29] K. H. Lee, “Sample Size Calculations with Dropouts in Clinical Trials,” Communications for Statistical Applications and Methods, vol. 15, no. 3, pp. 355–365, 2008.

[30] D. Muramatsu, A. Iwai, S. Aoki et al., “β-glucan derived from aureobasidium pullulans is effective for the prevention of influenza in mice,” PLoS ONE, vol. 7, no. 7. Article ID e41399, 2012.

[31] M. Jesenak, J. Majtan, Z. Rennerova, J. Kyselovic, P. Banovcin, and M. Hrubisko, “Immunomodulatory effect of pleuran (β-glucan from Pleurotus ostreatus) in children with recurrent respiratory tract infections,” International Immunopharmacology, vol. 15, no. 2, pp. 395–399, 2013.

[32] J. Pasnik, A. Spiel, A. Cwyinska-Bernas, K. Zeman, and M. Jesenak, “Preventive effect of pleuran (β-glucan from Pleurotus ostreatus) in children with recurrent respiratory tract infections - Open-label prospective study,” Current Pediatric Research, vol. 21, no. 1, pp. 99–104, 2017.

[33] V. Vettvicka, J. Richter, V. Svozil, L. R. Dobiašová, and V. Král, “Placebo-driven clinical trials of yeast-derived β-(1,3) glucan in children with chronic respiratory problems,” Annals of Translational Medicine, vol. 1, no. 3, article no. 26, 2013.

[34] J. E. McElhaney, V. Goel, B. Toane, J. Hooten, and J. J. Shan, “Efficacy of COLD-fX in the prevention of respiratory symptoms in community-dwelling adults: A randomized, double-blinded, placebo controlled trial,” The Journal of Alternative and Complementary Medicine, vol. 12, no. 2, pp. 153–157, 2006.

[35] National Institute of Food and Drug Safety Evaluation (NIFDS), Guidelines for Functional Evaluation of Health Functional Foods, ‘Can Help Improve Immune Function’, KFDA, Seoul, Korea, 2014.

[36] X. Zhou, M. S. Fragala, J. E. McElhaney, and G. A. Kuchel, “Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research,” Current Opinion in Clinical Nutrition & Metabolic Care, vol. 13, no. 5, pp. 541–547, 2010.

[37] H. O. Kim, H.-S. Kim, J.-C. Youn, E.-C. Shin, and S. Park, “Serum cytokine profiles in healthy young and elderly population assessed using multiplexed bead-based immunoassays,” Journal of Translational Medicine, vol. 9, article I13, 2011.

[38] A. Biancotto, A. Wank, S. Perl et al., “Baseline levels and temporal stability of 27 multiplexed serum cytokine concentrations in healthy subjects,” PLoS ONE, vol. 8, no. 12, Article ID e76091, 2013.

[39] D. L. McKay, G. Perrone, H. Rasmussen et al., “The Effects of a Multivitamin/Mineral Supplement on Micronutrient Status, Antioxidant Capacity and Cytokine Production in Healthy Older Adults Consuming a Fortified Diet,” Journal of the American College of Nutrition, vol. 19, no. 5, pp. 613–621, 2000.

[40] N. J. Hepburn, I. Garavita, E. A. Williams, D. R. Michael, and S. Park, “Probiotic supplement consumption alters cytokine and inflammatory marker measurements in clinical research,” Current Opinion in Clinical Nutrition, vol. 13, no. 5, pp. 541–547, 2010.

[41] W. L. Blok, J.-P. Deslypere, P. N. M. Demacker et al., “Probiotics and Infections,” Current Opinion in Gastroenterology, vol. 20, no. 2, pp. 438–445, 2010.

[42] D. M. Jesenak, J. Majtan, Z. Rennerova, J. Kyselovic, P. Banovcin, and M. Hrubisko, “Immunomodulatory effect of pleuran (β-glucan from Pleurotus ostreatus) in children with recurrent respiratory tract infections,” International Immunopharmacology, vol. 15, no. 2, pp. 395–399, 2013.