A randomized controlled trial to evaluate the effect of influenza vaccination and probiotic supplementation on immune response and incidence of influenza-like illness in an elderly population in Indonesia

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Abstract

Aim

To investigate the effect of influenza vaccination with or without probiotic supplementation on the immune response and incidence of influenza-like illness (ILI) in the elderly.

Methods

A randomized double-blind, placebo-controlled trial with a modified factorial design was conducted in 554 healthy elderly subjects aged 67 ± 5.6 (ranging from 60–90) years old in the Primary Health Care Center (Puskesmas area) of the Pulo Gadung District East Jakarta. Subjects received either a trivalent influenza vaccine or placebo at the start of the study, and a probiotic supplement (Lactobacillus helveticus R0052 and Lactobacillus rhamnosus R0011) or a placebo for 6 months. Subjects were randomly assigned into four intervention groups: influenza vaccine and probiotics (n = 141), influenza vaccine and placebo (n = 136), placebo and probiotics (n = 140), and both placebo (n = 137). The primary outcome was ILI incidence within 6 months. The secondary outcomes were seroprotection and seroconversion rates at 1, 4, and 6 months after administering the interventions.
Results
This study showed that the trivalent influenza vaccine increased seroprotection (RR 3.6 [95%CI 2.92–4.47]; p < 0.010) and seroconversion (RR 29.8 [95%CI 11.1–79.5]; p < 0.010) rates 1 month after vaccination in elderly people while the probiotic supplement did not alter influenza antibody titers (p = 1.000 and p = 0.210). The relative ILI incidence risk was similar between vaccinated and non-vaccinated groups, as well as in the probiotic group compared to the non-probiotic group.

Conclusion
The tested trivalent influenza vaccine significantly induced seroprotection and seroconversion in the vaccinated subjects, while probiotics administration did not influence these parameters. Vaccinated individuals displayed a similarly low ILI incidence as those in the Control Group. However, the observed trend towards a reduction of ILI incidence with probiotics supplementation warrants further assessments in a larger, at-risk population.

Clinical trial registry number
NCT03695432.

Introduction
Influenza is a major cause of mortality and morbidity worldwide [1]. Indeed, influenza viruses can cause only minimal symptoms, but also can lead to severe and lethal complications [2]. In general, influenza virus infections result in Acute Respiratory Illness (ARI). However, because ARI symptoms can also be caused by other infectious agents and are not specific to influenza viruses, this set of symptoms is referred to as Influenza-Like Illness (ILI) [3, 4]. In Indonesia, there is currently no available report on the prevalence of this disease. Based on symptoms used to define ARIs, prevalence is estimated at 25% [5]. Various studies have shown that influenza viruses and Respiratory Syncytial Virus (RSV) are often associated with acute respiratory disease requiring hospitalization, especially in the elderly population and patients with previous chronic disease [1, 6]. This is why individuals aged 65 years or older are considered among the most vulnerable groups, representing 90% of the reported cases of influenza-related complications.

Vaccination is considered as a primary preventive method in the management of influenza [7]. The efficacy of a vaccine at preventing disease can be inferred based on its efficacy and effectiveness at inducing seroconversion, conferring seroprotection, and reducing ILI incidence [8]. However, clinical studies on the effectiveness and efficacy of influenza vaccines in elderly populations have generated contradictory results [9].

Immunosenescence, which refers to the process of immune system aging that is reflected by an increased incidence of infections in the elderly, has been proposed as the cause underlying the reduced immunization response to vaccines observed in the elderly population. A new strategy is needed to improve the effectiveness of influenza vaccines in the elderly, either by improving the individuals’ immune response or vaccine formulations [10]. In this study, we explore whether probiotics can improve the immune response triggered by a trivalent influenza vaccine in the elderly, and reduce the incidence of ILI in this population.
Materials and methods

This study was a randomized, double-blind, placebo-controlled trial with a factorial design comparing the efficacy of two interventions, influenza vaccines and probiotics, at decreasing the risk of ILI in the elderly. The protocol of this study was approved by the Research Ethics Board of the Faculty of Public Health, University of Indonesia. The study protocol has also been registered in the Clinicaltrials.gov Registry, with the clinical trial registry number NCT03695432. There was a non-trial-related technical issue causing the delay in registering the study, that it was performed later after the subject enrollment started.

Eligible participants were randomized into four intervention groups: influenza vaccine + probiotics; influenza vaccine + placebo; placebo + probiotics; and both placebo. This study was conducted in the entire Pulo Gadung District, East Jakarta, between April and December 2015, which was the period encompassing flu season. At the beginning of this research, the dominant strain available was Strain B (lineage not determined), meanwhile Strain A(H1N1) pdm09 predominated later on. And the strains for the vaccines were A/California/7/2009 (H1N1)pdm09-like virus, A/Texas/50/2012(H3N2)-like virus, dan B/Massachusetts/2/2012-like virus.

Eligible subjects were healthy adults aged ≥60 years who presented themselves to vaccination and health education activities in various Primary Health Care Center (Puskesmas) of the East Jakarta district. In order to be enrolled in the study, potential participants had to have a BMI score between 17.5 and 29.9, and demonstrate a healthy mental state (MMSE score 28–30). Exclusion criteria were: contraindications to influenza vaccination, undergoing an immunomodulatory treatment in the past four weeks, immunosuppressant therapy, taking corticosteroids such as prednisone ≥ 20 mg/d for more than two weeks or for less than three months before the study. Potential participants were also excluded if they had previous influenza vaccination less than one year before the study, or were consuming probiotics (either manufactured or natural products) for more than seven days.

Screening was performed based on convenience sampling. Written informed consent was obtained from each subject prior to the trial-related screening. At baseline, blood samples were collected to measure basal influenza antibody levels. Then, eligible subjects were assigned into any of the four intervention groups, according to the randomization code provided by a contract research organization as the third party. The randomization code was generated by utilizing the Microsoft Excel® software. Subjects received the trivalent inactivated influenza vaccine (Flubio®, Biofarma, Bandung) or placebo (NaCl 0.9% solution) at the study initiation visit (month 0), with a supply of either Lacidofil® (Lactobacillus acidophilus R0052 and Lactobacillus rhamnosus R0011) or placebo. Therefore, all participants received similar interventions, that were the vaccine injection (or its placebo) and the oral probiotic capsules (or its placebo), in order to keep the investigator team, including the care providers and those assessed the outcomes, all subjects, as well as the laboratory personnel, blinded to the intervention allocation.

Follow-up visits were scheduled at 1, 4, and 6 months post-vaccination. The primary outcome was ILI incidence within 6 months. The secondary outcomes were seroprotection and seroconversion rates at 1, 4, and 6 months after administering the interventions. Compliance with probiotic supplementation was assessed by the study personnel of the Integrated Health Service Center (Posyandu), based on participant’s self-reported data.

To determine the efficiency of the influenza vaccine, the number of participants showing seroconversion or seroprotection was assessed. Participants with a baseline antibody titers <1:10 were considered seronegative at baseline, and a post-vaccination titer ≥ 1:40 was used to define seroprotection. A 4-times increase in the antibody titers after vaccination was required to infer seroconversion in those with a final titer having reached or exceeded 1:40.
Sample size was estimated based on projected ILI rates for the study target period. Our hypothesis was that the ILI incidence would be lower in the vaccinated group than in the corresponding placebo group. Similarly, we anticipated ILI incidence to be lower in the probiotics group than in the corresponding placebo group. Previously reported ILI rates in non-vaccinated elderly patients was 26.4% [4], and in vaccinated and probiotics-treated elderly individuals were 15.84% and 11.88%, respectively [4]. The ILI prevention effectiveness of the influenza vaccine was approximately 40% [11], and of probiotics was 55% [12]. Assuming a similar chance ($\pi$) to get ILI for all subjects in all groups at baseline (pre-vaccination), and using a significance level ($\alpha$) of 0.05, and a statistical power (1–$\beta$) of 80%, the minimum required sample size was 266 subjects for each group, estimated using the Freedman’s Equation below:

$$n = \frac{1}{(\theta_i + \pi) \left(\frac{\theta + 1}{\theta - 1}\right)^2 \left(Z_{1-\alpha} + Z_{1-\beta}\right)}$$

where $\theta$ refer to the expected hazard ratio, and $\pi$ is the chance to get ILI at baseline (pre-vaccination).

Therefore, for this two-by-two factorial design study, a total of 592 subjects were required, anticipating a drop-out rate of 10%. Each factorial group would require 148 subjects.

The rates of seroprotection and seroconversion as well as the ILI incidence between-group were statistically analyzed using chi-square test. While within-group analysis on seroprotection rate of each time-point after vaccination compared to the baseline (Pre-vaccination) was performed using cochrans-Q test. All statistical analysis was performed at a significance level ($\alpha$) of 0.05, 2-tailed. The analysis was performed using a statistical software Microsoft SPSS version 24.

**Results**

A total of 910 participants were screened between April and June 2015; of them, 280 were excluded for not meeting the inclusion criteria and the remaining 620 subjects were included and randomized. Of the remaining subjects, 554 completed the study and were included in subsequent analyses. Two primary interventions were studied: influenza vaccines and probiotic supplementation (Fig 1). Patient characteristics at baseline were similar between both interventions (Table 1) and between study groups (Table 2). Statistical analyses revealed no significant interaction between groups receiving vaccines and those receiving probiotics (OR 0.924, [95% CI 0.606–1.407]; $p = 0.712$).

The distribution of participants demonstrating seroconversion and seroprotection are presented in Table 4. There was a significant increase in post-vaccination seroprotection in groups receiving vaccines with probiotics and without probiotics at 1, 4, and 6 months, but it was not significant for groups who did not get vaccination (Table 3). Thus, in the proportion of seroconversion, there was no significant difference between the subjects who received vaccinations and probiotics compared to subjects have only received the vaccine at 1, 4, and 6 months (Tables 4 and 5).

Without considering probiotic supplementation, the relative risk (RR) associated with ILI incidence was similar between participants who received the influenza vaccine and those who received the vaccine placebo (RR = 1.0). When considering probiotic supplementation, the relative ILI incidence risk slightly lower (RR = 0.8) (Table 6). There was no significant difference in the relative ILI incidence risk according to intervention (Table 7).

Statistical analyses showed that the influenza vaccine did not reduce ILI relative risk (RR = 1.0) compared to non-vaccinated groups despite the positive effect of vaccination on the seroprotection status at 1, 4, and 6 months post-vaccination. Kaplan-Meier analysis for the
seroprotection (Fig 2) showed a significant difference in the maintenance of the seroprotection for 6 months between those who received the influenza vaccine and those who did not. The geometric mean titers (GMT) of the antibody anti-influenza over months are presented in Table 9. The antibody titers peaked out one month post-vaccination, and then gradually declined toward 6 months post-vaccination. The titer at Month 6 post-vaccination was still higher than that of pre-vaccination.

Of note, there was a non-significant reduction of the relative ILI risk (RR = 0.8) in participants receiving probiotics compared to those not receiving probiotics. However, probiotics administration did not influence the seroprotection and seroconversion status (Table 8).

**Discussion**

In most adults, a baseline level of pre-vaccination antibodies is detectable from ongoing influenza infections or previous vaccinations. Therefore, the humoral immune responses to certain viral strains in younger people are often different than in the elderly. As such, the post-vaccination antibody responses may strongly be affected by the priming process, without strictly representing the ability of the immune system.

The study in Hong Kong by Hui et al [13] also showed a relationship between seroprotection and influenza vaccination. The Hui et al study was based on a population with similar demographic and clinical characteristics as ours. Four weeks after vaccination, the seroprotection rate was 85.9% for H1N1 (OR = 8.1 [95% CI 0.6–47.8] p = 0.115) and 100% for Influenza B (p = 0.500). In our study, the seroprotection status after 1 month post-vaccination was significantly increased (chi-square = 83.101; p<0.010) as shown in Tables 3 and 4.

The outcomes of data analysis of this study in Table 5 show the seroconversion status. There was no significant increase among the intervention groups. Our statistical analyses showed that seroconversion rates were higher in the influenza vaccine + probiotics as well as in the influenza vaccine + placebo groups at the 1 month time point compared to the groups who did not receive the vaccine.
Table 1. Baseline characteristics of all participants included in the analyses, clustered by intervention.

| Characteristics          | Influenza Vaccine Intervention | Probiotic Intervention |
|--------------------------|--------------------------------|------------------------|
|                          | Vaccine (n = 277) | Non-Vaccine (n = 277) | p value  | Probiotic (n = 281) | Non-Probiotic (n = 273) | p value  |
| Age group                |                    |                        |          |                    |                        |          |
| 60–65 (n = 242[43.7])    | 130 (49.6)         | 112 (40.4)             | 0.573    | 60 (21.4)          | 52 (19.0)              | 0.649    |
| 66–70 (n = 144[26])      | 67 (24.2)          | 77 (27.8)              | 18 (6.4) | 21 (7.7)           |                        |          |
| 71–75 (n = 112[20.2])    | 53 (19.1)          | 59 (21.3)              |          | 11 (3.9)           |                        |          |
| > 80 (n = 17[3.1])       | 7 (2.5)            | 10 (3.6)               |          | 6 (2.2)            |                        |          |
| Gender                   |                    |                        |          |                    |                        |          |
| Male (n = 198[35.7])     | 97 (35)            | 101 (36.5)             | 0.732    | 99 (35.2)          | 99 (36.3)              | 0.800    |
| Female (n = 356[64.3])   | 180 (65)           | 176 (63.5)             | 182 (64.8) | 174 (63.7)     | 0.036                  |
| Marital Status           |                    |                        |          |                    |                        |          |
| Unmarried (n = 3[0.5])   | 3 (1.0)            | 0 (0)                  |          | 1 (0.4)           | 2 (0.7)                |          |
| Divorced (n = 246[44.4]) | 150 (54.2)         | 155 (56)               | 0.212    | 157 (55.9)        | 148 (54.2)             | 0.785    |
| Married (n = 305[55.1])  | 124 (44.8)         | 122 (44)               | 123 (43.7)| 123 (45.1)     |                        |          |
| Education                |                    |                        |          |                    |                        |          |
| High (n = 227[41])       | 110 (39.7)         | 117 (42.2)             | 0.545    | 103 (36.7)        | 124 (45.4)             | 0.036    |
| Low (n = 327[59])        | 167 (60.3)         | 160 (57.8)             | 178 (63.3)| 149 (54.6)     |                        |          |
| Number of Residents at Home |               |                        |          |                    |                        |          |
| Less than 4 (n = 271[48.9]) | 139 (50.2) | 132 (47.7)             | 0.552    | 131 (46.6)        | 140 (51.3)             | 0.272    |
| More than 4 (n = 283[51.1]) | 138 (49.8) | 145 (52.3)             | 150 (53.4)| 133 (48.7)     |                        |          |
| Nutritional Status       |                    |                        |          |                    |                        |          |
| Normal Body weight (n = 172[31.0]) | 83 (30) | 89 (32.1)              | 0.607    | 84 (29.9)         | 88 (32.2)              | 0.740    |
| Overweight (n = 275[49.6]) | 136 (49.1) | 139 (50.2)             | 144 (51.2)| 131 (48)       |                        |          |
| Obese (n = 107[19.3])    | 58 (20.9)          | 49 (17.7)              | 53 (18.9)| 54 (19.8)       |                        |          |
| Hypertension             |                    |                        |          |                    |                        |          |
| No (n = 247[44.6])       | 170 (61.4)         | 137 (49.5)             | 0.005    | 153 (54.4)        | 154 (56.4)             | 0.642    |
| Yes (n = 307[55.4])      | 107 (38.6)         | 140 (50.5)             | 128 (45.6)| 119 (43.6)     |                        |          |
| Diabetes mellitus        |                    |                        |          |                    |                        |          |
| No (n = 442[79.8])       | 220 (79.4)         | 222 (80.1)             | 0.832    | 220 (78.3)        | 222 (81.3)             |          |
| Yes (n = 112[20.2])      | 57 (20.6)          | 55 (19.9)              | 61 (21.7)| 51 (18.7)       | 0.375                  |
| Cardiovascular           |                    |                        |          |                    |                        |          |
| No (n = 50[9.0])         | 253 (91.3)         | 251 (90.6)             | 0.767    | 263 (93.6)        | 241 (88.3)             |          |
| Yes (n = 504[91])        | 24 (8.7)           | 26 (9.4)               | 18(6.4)  | 32 (11.7)       | 0.029                  |
| Cerebrovascular          |                    |                        |          |                    |                        |          |
| No (n = 54[97.5])        | 7 (2.5)            | 270 (97.5)             | 1.00     | 275 (97.9)        | 265 (97.1)             |          |
| Yes (n = 14[2.5])        | 7(2.5)             | 270 (97.5)             | 6 (2.1)  | 8 (2.9)         | 0.551                  |
| Chronic Pulmonary Disease|                    |                        |          |                    |                        |          |
| No (n = 524[94.6])       | 263 (94.9)         | 261 (94.2)             | 0.707    | 267 (95.0)        | 257 (94.1)             |          |
| Yes (n = 30[5.4])        | 14 (5.1)           | 16 (5.8)               | 14 (5.0) | 16 (5.9)       | 0.648                  |
| Exercise                 |                    |                        |          |                    |                        |          |
| ≥3x/week min. 30 min (n = 113[20.4]) | 55 (19.9) | 58 (20.9)              | 0.752    | 58 (20.6)        | 55 (20.1)              | 0.885    |
| < 3x/week min. 30 min (n = 441[79.6]) | 222 (80.1) | 219 (79.1)             | 223 (79.4)| 218 (79.9)     |                        |          |
| Smoking                  |                    |                        |          |                    |                        |          |
| Non-smoker (n = 391[70.6]) | 197 (71.1) | 194 (70)               | 0.780    | 203 (72.2)        | 188 (68.9)             | 0.383    |
| Smoker (n = 163[29.4])   | 80 (28.9)          | 83 (30)                | 78 (27.8)| 85 (31.1)       |                        |          |
| Vaccination History      |                    |                        |          |                    |                        |          |
| Yes (n = 19[3.4])        | 12 (4.3)           | 7 (2.5)                | 0.243    | 8 (2.8)         | 11 (4.0)               | 0.445    |

(Continued)
Furthermore, our data suggest that influenza vaccination with this trivalent vaccine was able to adequately stimulate the production of vaccine-specific antibodies at levels sufficient to induce seroprotection. Statistical analyses confirmed that the influenza vaccine intervention was significantly associated with seroprotection at 1 month, 4 months, and 6 months post-influenza vaccination along with a seroprotection potential approximately more than 3 times higher than in the non-vaccinated groups (RR > 3). Similarly, Kaplan-Meier analysis for the seroprotection (Fig 2) showed a significant difference in the maintenance of the seroprotection for 6 months between those who received the influenza vaccine and those who did not. This result was also corroborated by the marked elevation of antibody anti-influenza titer post-vaccination (Table 9). Apparently that even though 6 months after vaccination the antibody titer had declined to a level that was no longer significantly different with that of the pre-vaccination, the titer was still adequate to yield an effective protection for the subjects. A similar scenario was observed at the 1 month time point; a seroconversion ability of 43% is above the accepted standard of > 30% for successful influenza vaccines for the elderly.

However, there was no significant difference in ILI incidence between the vaccinated and control groups, with a p value = 1.000, a relative ILI incidence risk (RR) of 1.0, and a null Absolute Risk Reduction (ARR) factor. The fact that very few participants experienced ILI during the course of the study precludes any strong conclusions about the true effectiveness of this vaccine against influenza infections. Indeed, statistical analyses that had been conducted to find the relationship between vaccines and ILI, have shown that the influenza vaccines did not provide a significant level of protection against ILI, with a RR = 1, which means that participants in both the vaccine and placebo groups have a relatively similar risk of developing ILI. This could be attributable to our sampling strategy. As our study population was recruited...
Table 2. Participant characteristics in the four intervention groups.

| Variable                | Category n (%) | Influenza Vaccine and Probiotics (n = 141) | Influenza Vaccine and Placebo (n = 136) | Placebo and Probiotics (n = 140) | Both Placebo (n = 137) | P Value |
|-------------------------|----------------|-------------------------------------------|----------------------------------------|---------------------------------|------------------------|---------|
|                         |                | N %                                      | N %                                    | N %                             | N %                    |         |
| Median                  |                | 66 (60–85)                               | 66 (60–86)                             | 67 (60–90)                      | 67 (60–85)             | 0.838   |
| Age                     | 60–65 (n = 242) | 66 46.8                                   | 64 47.1                                | 57 40.7                         | 55 46.8                |         |
|                         | 66–70 (n = 144) | 31 22.0                                   | 36 26.5                                | 38 27.1                         | 39 22.0                |         |
|                         | 71–75 (n = 112) | 29 2.6                                    | 24 17.6                                | 31 22.1                         | 28 2.6                 |         |
|                         | 76–80 (n = 39)  | 11 7.8                                    | 9 6.6                                  | 7 5.0                           | 12 7.8                 |         |
|                         | > 80 (n = 17)   | 4 2.8                                     | 3 2.2                                  | 7 5.0                           | 3 2.8                  |         |
| Gender                  | Men (n = 198)   | 45 31.9                                   | 52 38.2                                | 54 38.6                         | 47 31.9                | 0.598   |
|                         | Women (n = 356) | 96 68.1                                   | 84 61.8                                | 86 61.4                         | 90 68.1                |         |
| Status                  | Not married    | 1 0.7                                     | 2 1.5                                  | 0 0                             | 0 0                    | 0.675   |
|                         | Divorced       | 63 44.7                                   | 61 44.9                                | 60 42.9                         | 62 44.7                |         |
|                         | Married        | 77 54.6                                   | 73 53.7                                | 80 57.1                         | 75 54.6                |         |
| Education               | High (n = 227)  | 46 32.6                                   | 64 47.1                                | 57 40.7                         | 60 32.6                | 0.086   |
|                         | Low (n = 305)   | 95 67.4                                   | 72 52.9                                | 83 59.3                         | 77 67.4                |         |
| Number of Resident      | Less than 4    | 76 53.9                                   | 62 45.6                                | 74 52.9                         | 71 53.9                | 0.513   |
|                         | More than 4    | 65 46.1                                   | 74 54.4                                | 66 47.1                         | 66 46.1                |         |
| Nutritional Status      | Normal         | 45 31.9                                   | 38 27.9                                | 39 27.9                         | 50 31.9                | 0.603   |
|                         | Overweight     | 67 47.5                                   | 69 50.7                                | 77 55.0                         | 62 47.5                |         |
|                         | Obese          | 29 20.6                                   | 29 21.3                                | 24 17.1                         | 25 20.6                |         |
| Hypertension            | No             | 82 58.2                                   | 88 64.6                                | 71 50.7                         | 66 48.2                | 0.025   |
|                         | Yes            | 59 41.8                                   | 48 35.3                                | 69 49.3                         | 71 51.8                |         |
| Diabetes Mellitus       | No             | 109 77.3                                  | 111 81.6                               | 111 79.3                        | 111 81                 | 0.808   |
|                         | Yes            | 32 22.7                                   | 25 18.4                                | 29 20.7                         | 26 19                  |         |
| Cardiovascular          | No             | 133 94.3                                  | 120 88.2                               | 130 92.9                        | 121 88.3               | 0.175   |
|                         | Yes            | 8 5.7                                     | 16 11.8                                | 10 7.1                          | 16 11.7                |         |
| Cerebrovascular         | No             | 139 98.6                                  | 131 96.3                               | 136 97.1                        | 134 97.8               | 0.669   |
|                         | Yes            | 2 1.4                                     | 5 3.8                                  | 4 2.9                           | 3 2.2                  |         |
| Lungs                   | No             | 134 95                                   | 129 94.9                               | 133 95                          | 128 93.4               | 0.924   |
|                         | Yes            | 7 5.0                                     | 7 5.1                                  | 7 5.0                           | 9 6.6                  |         |
| Physical Exercise       | ≥ 3x/week min. | 27 19.1                                   | 28 20.6                                | 31 22.1                         | 27 19.7                | 0.932   |
|                         | < 30x/week min.| 114 80.9                                  | 108 79.4                               | 109 77.9                        | 110 80.3               |         |
| Smoking History         | Not smoking    | 106 75.2                                  | 91 66.9                                | 97 69.3                         | 97 70.8                | 0.488   |
|                         | Smoking        | 35 24.8                                   | 45 33.1                                | 43 30.7                         | 40 29.2                |         |
| Vaccine History         | Vaccines       | 6 4.3                                     | 6 4.4                                  | 2 1.4                           | 5 3.6                  | 0.494   |
|                         | Non-vaccines   | 135 95.7                                  | 130 95.6                               | 138 98.6                        | 132 96.4               |         |
| Dependency              | Independent    | 130 92.2                                  | 123 90.4                               | 131 93.6                        | 129 94.2               | 0.651   |
|                         | Dependent      | 11 7.8                                    | 13 9.6                                 | 9 6.4                           | 8 5.8                  |         |
| Frailty Index           | Non-frail      | 90 63.8                                   | 77 56.6                                | 91 65                           | 94 68.6                | 0.215   |
|                         | Frail          | 51 36.2                                   | 59 43.4                                | 49 35                           | 43 31.4                |         |
| Depression Level        | Not depressed  | 116 82.3                                  | 111 81.6                               | 116 82.9                        | 115 83.9               | 0.964   |
|                         | Depressed      | 25 17.7                                   | 25 18.4                                | 24 17.1                         | 22 16.1                |         |
| Seroprotection          | Seroprotection | 45 31.9                                   | 39 28.7                                | 35 25                           | 40 29.2                | 0.645   |
|                         | No seroprotection | 96 68.1                              | 97 71.3                                | 105 75                          | 97 70.8                |         |

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from people attending to health education activities in various Primary Health Care Center (Puskesmas) of the East Jakarta district, it is likely that the uptake of lifestyle- and hygiene-related ILI prevention measures provided through the health education activities was successful in this specific population, thereby reducing the overall ILI incidence in our study sample.
With regards to the probiotics intervention, there was a tendency towards a higher seroconversion status at the 1- and 4-month time points in the vaccine + probiotics supplementation group compared to the vaccine + placebo group, suggesting a potential role for probiotics role in enhancing seroconversion. As our study appeared to have been underpowered to detect ILI incidence in this particular segment of the population, this trend warrants further studies to establish the effect of probiotics supplementation on relative ILI risk in the elderly.

**Conclusions**

The tested influenza vaccines significantly induced seroprotection and seroconversion in an elderly population. However, as the overall relative risk of ILI events was low in our population, no reduction in the relative risk of ILI events was observed in vaccinated individuals. While probiotic supplementation did not influence seroprotection and seroconversion in our study population, the observed trend towards a reduction in ILI incidence warrants for further assessments in a larger, at-risk population.
Table 8. ILI incidence, seroprotection, and seroconversion in influenza vaccine intervention (vaccine vs non-vaccine) and in probiotic intervention (probiotic vs non-probiotic).

|                  | Influenza Vaccine | Probiotics |
|------------------|------------------|------------|
|                  | Vaccine (n = 277) n (%) | Non-Vaccine (n = 277) n (%) | RR (95% CI) | P Value | Probiotics (n = 281) n (%) | No Probiotics (n = 273) n (%) | RR (95% CI) | P Value |
| ILI              | 9 (3.2)          | 9 (3.2)    | 1.0 (0.40–2.48) | 1.000   | 8 (2.8)          | 10 (3.7)    | 0.8 (0.31–1.940) | 0.588   |
| No ILI           | 268 (96.8)       | 268 (96.8) |                     |         | 273 (97.2)       | 263 (96.3)  |                     |         |
| Seroprotection 0 month |
| Seroprotection   | 84 (30.3)        | 75 (27.1)  | 1.1 (0.86–1.46)    | 0.398   | 80 (28.5)        | 79 (28.9)   | 1.0 (0.76–1.28)  | 0.903   |
| No Seroprotection| 193 (69.7)       | 202 (72.9) |                     |         | 201 (71.5)       | 194 (71.1)  |                     |         |
| Seroprotection 1 month |
| Seroprotection   | 257 (92.8)       | 72 (26)    | 3.6 (2.92–4.47)    | < 0.010 | 168 (59.4)       | 163 (59.3)  | 1.0 (0.87–1.15)  | 1.000   |
| No Seroprotection| 20 (7.2)         | 205 (74)   |                     |         | 115 (40.6)       | 112 (40.7)  |                     |         |
| Seroprotection 4 months |
| Seroprotection   | 229 (82.7)       | 68 (24.5)  | 3.3 (2.72–4.17)    | < 0.010 | 151 (53.7)       | 146 (53.5)  | 1.0 (0.86–1.17)  | 1.000   |
| No Seroprotection| 48 (17.3)        | 209 (75.5) |                     |         | 130 (46.3)       | 127 (46.5)  |                     |         |
| Seroprotection 6 months |
| Seroprotection   | 204 (73.6)       | 57 (20.6)  | 3.6 (2.81–4.56)    | < 0.010 | 134 (47.7)       | 127 (46.5)  | 1.0 (0.86–1.22)  | 0.849   |
| No Seroprotection| 73 (26.4)        | 220 (79.4) |                     |         | 147 (52.3)       | 146 (53.5)  |                     |         |
| Seroconversion 1 month |
| Seroconversion   | 119 (43)         | 4 (1.4)    | 29.8 (11.1–79.5)   | < 0.010 | 69 (24.6)        | 54 (19.8)   | 1.2 (0.91–1.7)   | 0.211   |
| No Seroconversion| 158 (57)         | 273 (98.6) |                     |         | 212 (75.4)       | 219 (80.2)  |                     |         |
| Seroconversion 4 months |
| Seroconversion   | 63 (22.7)        | 4 (1.4)    | 15.8 (5.81–42.7)   | < 0.010 | 37 (13.2)        | 30 (11.0)   | 1.2 (0.76–1.88)  | 0.512   |
| No Seroconversion| 214 (77.3)       | 273 (98.6) |                     |         | 244 (86.6)       | 243 (89.0)  |                     |         |
| Seroconversion 6 months |
| Seroconversion   | 40 (14.4)        | 3 (1.1)    | 13.3 (4.17–42.6)   | < 0.010 | 21 (7.5)         | 22 (8.1)    | 0.9 (0.52–1.65)  | 0.920   |
| No Seroconversion| 237 (85.6)       | 274 (98.9) |                     |         | 260 (92.5)       | 251 (91.9)  |                     |         |

The trivalent influenza vaccine included A/California/7/2009(H1N1)pdm09-like virus, A/Texas/50/2012(H3N2)-like virus, dan B/Massachusetts/2/2012-like virus strains.

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Table 9. Geometric Mean Titers (GMT) of the antibody anti-influenza over months.

| GMT values | Influenza Vaccine | Probiotics |
|------------|------------------|------------|
|            | Vaccine (n = 277) | Non-Vaccine (n = 277) | P value | Probiotics (n = 281) | No Probiotics (n = 273) | P value |
| Month 0 (pre-vaccination) | 24.7826 | 25.4143 | 0.700 | 26.4467 | 23.8151 | 0.900 |
| Month 1 post-vaccination   | 297.0046 | 27.2839 | <0.001 | 97.7981 | 82.8590 | 0.800 |
| Month 4 post-vaccination   | 130.5249 | 25.8472 | 0.008 | 40.9193 | 54.2132 | 0.150 |
| Month 6 post-vaccination   | 99.1869 | 24.2842 | 0.500 | 56.1377 | 42.9065 | 0.400 |

The trivalent influenza vaccine included A/California/7/2009(H1N1)pdm09-like virus, A/Texas/50/2012(H3N2)-like virus, dan B/Massachusetts/2/2012-like virus strains.

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Supporting information

S1 File. This is the anonymised raw data of antibody titre of the study subjects. (PDF)

S2 File. This is the study protocol in original version (in Bahasa Indonesia). (PDF)
S3 File. This is the study protocol in translated version (in English).
(PDF)

S1 Checklist. This is the consort checklist of the manuscript.
(DOC)

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