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EFFECTIVE SYMPTOMATIC TREATMENT AND REDUCTION OF INFLUENZA VIRAL LOAD IN RESPIRATORY INFECTED CHILDREN BY NASAL-SPRAYING *BACILLUS* SPORE PROBIOTICS (LIVESPO® NAVAX)

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ABSTRACT:

The influenza virus is a common cause of respiratory tract infections in children, and the treatment is mainly symptomatic reduction. We conducted a blind, randomized and controlled clinical trial to investigate the effects of LiveSpo® Navax containing ≥ 5 billion *Bacillus* spores in symptomatic treatment for children with acute respiratory diseases caused by the influenza virus at the Vietnam National Children's Hospital. A total of 30 influenza-infected patients (*n* = 15/group x 2 groups: Navax and Control) participated in the study, they were nasal-sprayed 3 times a day in conjunction with standard hospital drugs. As a result, recovery time for fever and runny nose in the Navax group were 1 to 2-day shorter than the control group, equivalent to more effectiveness in Navax group by about 53-90% in in comparison to the control group. After 2 days of treatment, the group of patients using LiveSpo® Navax showed effectively reduction in influenza viral load (900-fold reduction), which was 60-fold higher than those in control group (15-fold reduction). The Navax group showed neither abnormal signs of breathing nor irritation in the nasal mucosa. This primary clinical study demonstrates the safety, symptomatic-reduction, and viral load-relieving effects of *Bacillus* spore probiotics as a nasal sprays in children infected with the influenza virus.

Keywords: LiveSpo® Navax , *Bacillus* spore probiotics, influenza virus, respiratory infection, children.

I. INTRODUCTION

Influenza viruses (including influenza A (subtypes A/H1, A/H3, A/H5) and influenza B), characterized by single strain RNA, are the most common pathogens causing Acute Respiratory Tract Infection (ARTI) in children such as nasopharyngitis, pneumonia, laryngitis, bronchiolitis, high fever, runny nose. The disease could progress rapidly and severely with respiratory failure, difficulty breathing, dry rales and moist rales [1,2]. Influenza viruses persist year-round with annual outbreaks in Vietnam, peaking in winter-spring transition (at about November to

December). In the world, about 20% of children get flu every year [3]. The climate change is one of the causes of variation in infection level and duration of influenza infection [4]. As estimated by US CDC, in the year 2015, there were 17.2 billion of respiratory infection cases around the world. Influenza viruses are highly contagious by getting into the eyes, nose, mouth or easily spreading through the air via respiratory droplets of infected person such as cough, sneeze or talk [5,6]. According to recent report (Nov-2020), the number of hospitalized children due to respiratory infections in National Children's Hospital (Hanoi) is twice as high as normal, of which nearly 10% were flu-infected children. Only in November 2020, about 500 flu-infected patients were hospitalized, showing 10-20% increase compared to the previous months. The hospitalized children often showed difficulty breathing, respiratory failure with possible complications include pneumonia, bronchiolitis, otitis media or viral re-infection (7).

Related to prevention and treatment of flu, the prophylactic vaccines have been used for many years and ostelamivir drug has achieved good treatment effect. However, influenza viruses change frequently, requiring a yearly booster vaccination while it may cause unwanted side effects [8]. The treatment method using ostelamivir on children also not recommended for severe cases because of side effects [9,10]. Recently, prophylactic treatment for respiratory tract infections has been increasingly improved, in which probiotics are considered as promissing candidates for therapeutic support and reduction of anti-biotic dependence [11,12]. Probiotics are microorganisms, which are safe and beneficial for human health. The recent studies showed that alonging with being a beneficial bacteria for digestive system, probiotics are also able to prevent the respiratory tract infections due to ability to absorb and inactivate the viruses, produce secondary substances that inhibit virus growth and stimulate the immune system to capture and kill the invased viruses. However, most of probiotic products is oral administration and the effects obtained on respiratory tract is quite slow, normally after 3-12 months [13].

The effects of nasal-spraying probiotic product LiveSpo® Navax containing 5 billion of live spores *Bacillus subtilis* ANA4 and *Bacillus clausii* ANA39/5 mL was evaluated on RSV-infected children. The results showed that Navax treatment resulted in 1-day faster recovery time and 300-fold reduction in RSV load at day 3 of treatment regimen [14]. In this study, we initially investigated the safety and efficacy of LiveSpo® Navax on supporting symptomatic treatment in children infected with influenza virus being treated at National Children's Hospital during 6 treatment days. Besides, we evaluated the viral load reduction level and the presence of two strains *B. subtilis* ANA4 and *B. clausii* ANAA39 in nasopharyngeal samples of patients.

II. METHODS

2.1. SUBJECTS

The pediatric patients were from 4-60 months in age, volunteered to participate in the study and being diagnosed bronchiolitis with flu-positive.

The specialized product LiveSpo® Navax (Registration number: 210001337/PCBA-HN) manufactured by LiveSpo Pharma Co. Ltd., which is prepared in a form of 0.9% NaCl

physiological saline suspension containing probiotic spores of two bacterial strains *B. subtilis* ANA4 and *B. clausii* ANAA39 with extremely high purity, high concentration (5 billion CFU/5 mL). Product is use as nasal-spraying, directly spray into the nose or throat.

2.2. Time and place of study

The study was performed in International Department S and Department of Molecular Biology for Infectious Diseases, National Children's Hospital from March, 15th, 2021 to December, 30, 2021.

2.3. Study design

The blind, randomized and controlled clinical trial on two groups of patient as showed in Figure 1.

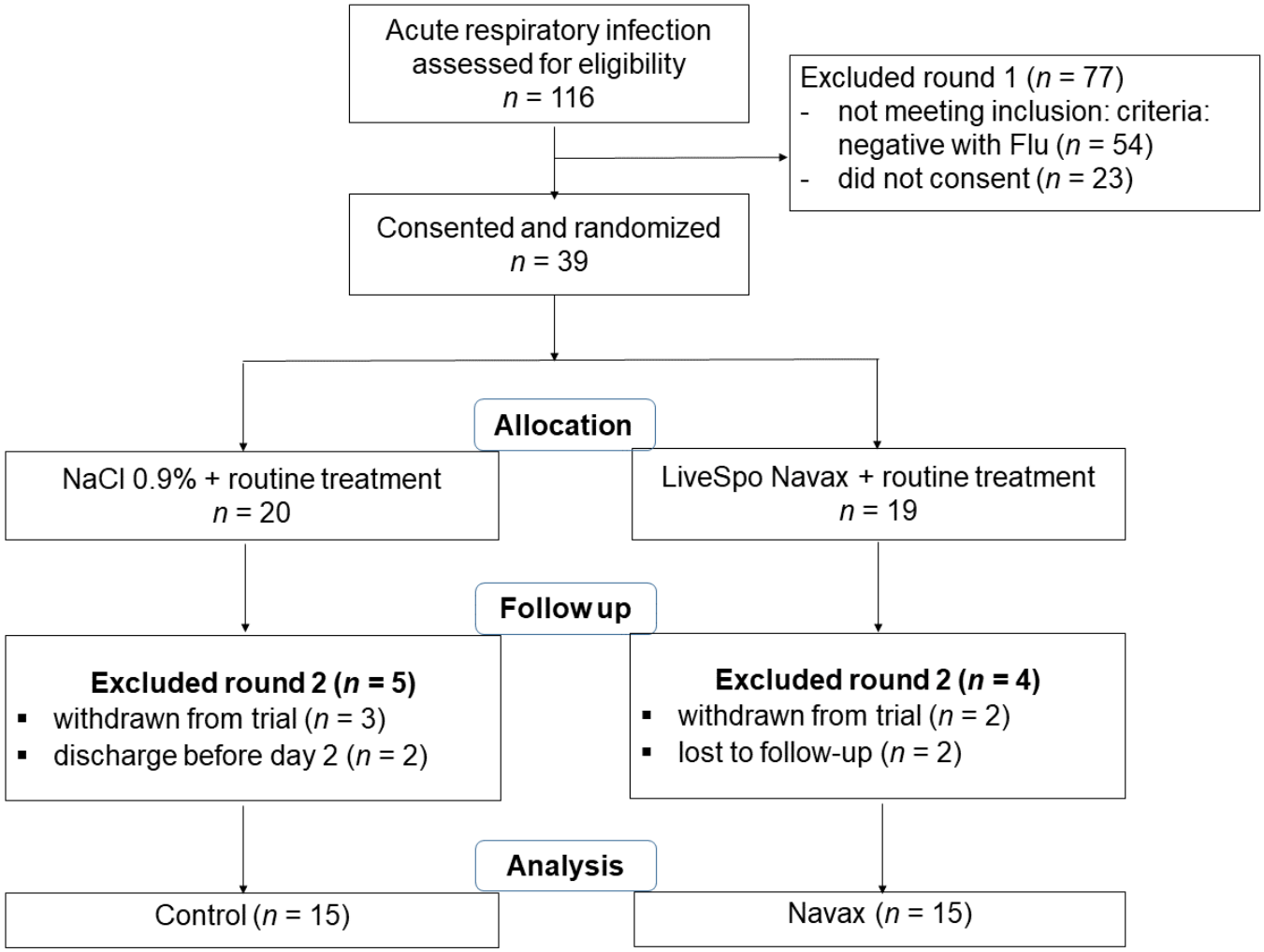

Figure 1: Diagram displaying the flow of participants involved in the study

2.4. Sample size

The sample size of randomized clinical trial on two groups was calculated based on a hypothesis is that LiveSpo® Navax alleviate flu-infection symptoms about 35% more effectively, as indicated by 95% of patients in the Navax group are symptom free at day 3 or 4 of intervention, compared to 60% of patients in the control group (depending on symptoms). Based on this hypothesis, the random sample size was calculated according to the following formula:

$$n = \frac{\left[z_\alpha \sqrt{2p(1-p)} + z_\beta \sqrt{p_1(1-p_1) + p_2\,(1-p_2)}\right]^2}{(p_1 - p_2)^2}$$

In there, Z_α = 1.96 (α = 0.05); Z_β = 0.84 (power level = 0.8); p_1 in control group = 0.60, p_2 in Navax group = 0.95, $p = p_1 + p_2)/2 = 0.775$; n_1 in control group and n_2 in Navx group are equal, $n_2/n_1=1$. Estimated required sample size for each group was 21, equivalent to 42 infected patients in total. Due to the percentage of hospitalized children in International Center S, National Children's Hospital caused by acute respiratory diseases with flu-positive is about 30%, about 116 patients were estimated for screening. However, the time of study coincided with the pandemic COVID-19, causing the very low number of flu-patients hospitalized, only 39 patients were collected in total, in which groups of 20 and 19 patients were assigned randomly into Navax group and control group, respectively. During study, some of the patients withdrew, the number of patients participated until the end of trial was 15 patients/group. The research diagram was described in Figure 1.

2.5. Sample selection method

Samples were selected according to convenience sampling principle. Patients were selected and randomly assigned into two group by drawing number 0 and 1: Patients who had 0 number were belong to group treated by 0.9% physiological saline (control group); patients who had 1 number were belong to group treated with LiveSpo® Navax (Navax group).

2.6. Study variations

The variations of study include:

The common characteristics of research subjects: Age, gender.

Clinical characteristics prior to treatment: Runny nose, fever, fast pulse, fast breath.

Sub-clinical characteristics: Cardiopulmonary X-Ray, hematological indexes, serum C-reactive protein (CRP), influenza viral concentration.

2.7. Study procedure

Diagnosis and screening of patients with influenza virus infection from nasopharyngeal samples by rapid test method using a commercial kit "BV Veritor System for rapid detection of Flu A+B (Becton Dickison)"; blood test, serum C-reaction protein (CRP) and total white blood

cells help determine the infection level; cardiopulmonary X-Ray at day 0 was performed in International Department S, National Children's Hospital; Influenza viral quantification by real time RT-PCR TaqMan Probe for nasopharyngeal samples according to routine test at Department of Molecular Biology for Infectious Disease – National Children's Hospital (ISO 15189).

Study was conducted in parallel on 2 groups of patients who were given nasal-spraying probiotic LiveSpo® Navax and 0.9% NaCl physiological saline solution, coded in form of blind sample to ensure the objectivity of study. Product was assigned to use immediately after sub-grouping, in parallel with the usual treatment regimen at the hospital. Because of LiveSpo® Navax is odorless, tasteless and contained in opaque plastic spray bottle, so the nurses can't distinguish between probiotic suspension and placebo. The nurses were guided to use the spray bottle at about 50 µl/time x 3 times a day, spraying directly into the nasal cavity in continuously 6 days along with routine treatment drugs at the hospital, which includes the following oral drugs: Paracetamol for fever reduction (Efferegant®), Carbocysteine for expectoration (Carbothiol®), Oseltamivir phosphate for anti-virus (Tamiflu®) or anti-biotic drugs such as Cefotaxim (Goldcefo®), Amoxiciline/clavulanic acid (Augmentin®) used in cases of superinfection with bacteria due to anti-biotic susceptibility test results. Each 50µl LiveSpo® Navax contains ≥ 2.5 x 10^8 CFU spores of *B. subtilis* ANA4 and *B. clausii* ANAA39 in total. During treatment period, patients were monitored daily for typical clinical symptoms of respiratory tract infection cause by influenza virus, including: runny nose, fever, dry rales, moist rales, pulse and breath among 6 days. Nasopharyngeal samples in both groups were prepared by solid plastic handle with breakpoint swab PurFlock Ultra (Puritan, US), then soak the cotton swab in 1 mL of 0.9% NaCl physiological saline solution to collect the suspension containing virus for evaluation of threshold cycle (C_t) of influenza virus in nasopharyngeal samples at 2 time points: Day 2 (after 48 hours of routine treatment in combine with product) and day 0 (before treatment with product) by real time PCR/RT-PCR Taqman probe. The presence of *B. subtilis* ANA4 and *B. clausii* ANA39 in nasopharyngeal sample at day 2 was detected by Real-time PCR SYBR Green using primers specifically to two species *B. subtilis* and *B. clausii*, which was optimized according to ISO 17025 standard at Key Laboratory of Enzyme and Protein Technology (KLEPT), Hanoi University of Science. The specific sequence of primers and probe for influenza virus, *B. subtilis*, *B. clausii* and internal standard gene (β-actin) were described in Table 1.

The efficacy of LiveSpo® Navax was evaluated according to semi-quantitative assays for measuring changes in influenza viral load after 2 days of treatment and decrease level of clinical and sub-clinical symptoms. In which, the decrease level of viral load was calculated by $2^{\Delta Ct}$ formula (ΔC_t = C_t of day 2 – C_t of day 0 [15]; C_t is the value of threshold cycle of RT-PCR Taqman probe reaction, specifically for influenza viruses. The quantity of *B. subtilis* and *B. clausii* in nasopharyngeal sample was expressed by C_t value of Real-time PCR SYBR Green reaction, specifically for these two probiotic strains. In order to confirm that the fluorescent signal SYBR Green in Real-time PCR reaction is due to specific amplification, the melting

curves and melting temperature (T_m) of each sample were analyzed and calculated, then compared to T_m of standard *B. subtilis* (85°C + 1) and *B. clausii* (84°C + 1).

Table 1: Specifically primers and probe for influenza virus, internal standard gene of RT-PCR Taqman probe (CDC) and primers for *B. subtilis* and *B. clausii* of Real-time PCR SYBR Green

Primers/Probe	Sequence (5' - 3')	Quantity (pmol/reaction)
Influenza virus		
A-Fw	GAC CRA TCC TGT CAC CTC TGA C	5
A-Rv	AGG GCA TTY TGG ACA AAK CGT CTA	5
A-FAM probe	/56FAM/-TGC AGT CCT CGC TCA CTG GGC ACG-(BHQ1)	1
B-Fw	TCC TCA ACT CAC TCT TCG AGC G	5
B -Rv	CGG TGC TCT TGA CCA AAT TGG	5
B-JOE probe	(JOE)-CCA ATT CGA GCA GCT GAA ACT GCG GTG-(BHQ1)	1
H1N1-Fw	AAG CAA CAA AAA TGR AGG CAA TAC TA	5
H1N1-Rv	TCT GTT GAA TTG TTC GCA TGA TAA	5
H1N1-FAM probe	/56FAM/-TTR CAA CCG CAA ATG CAG ACA CAT TAT G-(BHQ1)	1
H3N2-Fw	AAG CAT TCC YAA TGA CAA ACC	5
H3N2-Rv	ATT GCR CCR AAT ATG CCT CTA GT	5
H3N2-JOE probe	(JOE)-CAG GAT CAC ATA TGG GSC CTG TCC CAG-(BHQ1***a***)	1
H5N1-Fw	ACATGCCCAAGACATACTGGAA 130	5
H5N1-Rv	GAATTCGTCACACATTGGGTTTC	5
H5N1-Cy5 probe	Cy5/CAC ACA ACG/TAO/GGA AGC TCT GCG	1

ATC T/3IABkFQ/

Internal standard		
β-actin-Fw	GATGTCCACGTCACACTT CA	5
β-actin-Rv	ATGCCTGAGAGGGAAATGAGGGC	5
β-actin-ROX probe	5'-HEX-ATGCCTGAGAGGGAAATG AGG GC-BHQ2-3	1
Bacillus		
subtilis-Fw	ACCATTGCGGTAGGTGCG	5
subtilis-Rv	GCGTTTGTCCAAGTCGGG	5
clausii-Fw	AATTTTTACCGCCCCTCAAG	5
clausii-Rv	ACTTTTGGAACATGCCGAAC	5

2.8. Data collection method

The general information such as history of hospitalization, nutrient, history of drugs usage… of patients was collected by the questionnaire in individual medical record. Moreover, clinical symptoms during 6 days of treatment (runny nose, body temperature, pulse and breath, vomiting and diarrhea (if any), pulse oximetry (SpO_2) and other sub-clinical indexes at certain time points (cardiopulmonary X-ray; total while blood cells; CRP; C_t of influenza virus, *B. subtilis* and *B. clausii)* were monitored and filled into patients' medical record. Each patient's information was then gathered and systematized in a data sheet for analysis.

2.9. Statistical analysis

Data analysis and processing was carried out according to medical statistical assay. Tabular analysis of demographic and clinical/sub-clinical difference before treatment in two groups was performed using the Fisher's Exact Test when the expected value of any cell was below 5 (Table 2). Distribution of the data was verified using both the normality test and QQ plot. Variables were compared using Mann-Whitney test (Figure 2A, 3). Statistical and graphical analyzes were performed on GraphPad Prism v8.4.3 software (GraphPad Software, US). The significance level of all analyzes was set at the $p< 0.05$ with $*p<0.05$, $***p<0.001$ and $****p<0.0001$.

2.10. Ethical issues

This study received the ethics approval by the Ethics Committee in Medical Research of the Vietnam National Children's Hospital under Decision No. 441/BVNTW-VNCSKTE on

March, 11th 2021.

III. RESULTS

3.1. The characteristics of patients prior to treatment

The collected data on 30 patients with flu-positive before treatment showed that the clinical symptoms in both groups include: (i) 100% (15/15) of patients had runny nose, (ii) 77% of patients had fever, 80% of patients had fast pulse, 16.7% of patients fast breath; the sub-clinical symptoms include: 70% of patient had high CRP level (> 6 mg/L), 57% of patients had high total while blood cells (>10.0 g/L). The X-ray images showed that most of patients (87%) had lung lesion (osler's nodes, hyperinflation). Clinical and sub-clinical characteristics of patients in Navax and Control groups were summarized in Table 2 below. It demonstrated that the research subjects are bronchiolitis patients with similar characteristics (no statistical difference, p>0.05).

Table 2: Clinical and sub-clinical characteristics in flu-infected children before treatment

Indexes	**Flu-infected patients**		
	Navax group (*n* = 15)	**Control group (*n* = 15)**	***p***
Age (month) *n* (%)			
≥ 4 -12	2 (13.33)	4 (26.67)	0.37
> 12	13 (86.67)	11 (73.33)	
Gender *n* (%)			
Male	9 (60)	11 (73.33)	0.44
Female	6 (40)	4 (26.67)	
Clinical characteristic *n* (%)			
Runny nose	15 (100)	15 (100)	1.00
Fast pulse (beats/min)			
≥ 4 -12 months (>140). n (%)	1 (6.67)	1(6.67)	1.00
> 12 months (>120). n (%)	11 (73.33)	11 (73.33)	1.00
Fast breath (beats/min)			
≥ 4 -12 months (>40). n (%)	0 (0.00)	0.00)	1.00

> 12 months (>32). n (%)	3 (20)	2 (13.33)	0.63
Fever (>37.5℃)	13 (86.67)	10 (66.67)	0.21
Average hospitalized days	2,3	3,0	0.11
Sub-clinical characteristic *n* (%)			
Cardiopulmonary X-ray showed oster's nodes, hyperinflation...	13 (86.67)	13 (86.67)	1.00
Total while blood cells >10.0 G/L	7 (46.67)	10 (66.67)	0.27
CRP > 6.0 mg/L	9 (60)	12 (80)	0.24

3.2. Safety and symptomatic-relieving effects of LiveSpo® Navax on children with influenza infection

During the entire treatment period, we did not record any case with abnormal changes of breathing rate, pulse, body temperature and pulse oximetry (SpO_2) upon spraying LiveSpo® Navax. The breathing rate, pulse, body temperature and SpO_2 in both groups were fluctuated around 1 beat/min, 3 beats/min, 0.5°C, 0.5%, respectively while all other survival indicators were all within allowable limits. 100% of patients participating in the trial showed no signs of nasal mucosa irritation or digestive disorders such as vomiting, diarrhea during the treatment. That means using nasal-spraying spore probiotics *Bacillus* did not cause any side effects on flu-infected children. Patients in Navax groups have a faster reduction of clinical symptoms than those in control group. Specifically, with 95% CI for median, the results in Figure 2A showed that the recovery time of runny nose (median Navax/0.9% NaCl: 3 days/5 days; 95% CI: 2-3 days/3-6 days) and fever (median Navax/0.9% NaCl: 2 days/3 days; 95% CI: 2-3 days/2-4 days) in Navax group was faster than control group by 2 days and 1 days, respectively. This study also showed that the days of treatment corresponded to 50% cured patients (DT_{50}) for typical symptoms such as runny nose and fever observed in Navax group ware 2.2 and 1.5 days, respectively (Figure 2B), showing the shorter value in comparison to control group (4.2 and 2.3 days, respectively) (Figure 2C). That means the treatment effectiveness in Navax group was about 53-90% higher than control group. We concluded that nasal-spraying *Bacillus* spores (Navax group) help reducing the typical symptoms of flu by 1-2 recover days earlier and 53-90% higher efficacy than 0.9% NaCl (control group) with statistically significant difference (*$p<0.05$). We did not found the shorten efficacy of LiveSpo® Navax on treatment time of fast pulse

(median Navax/0.9% NaCl: 3 days/3 days; 95% CI: 2-3 days/3-4 days) and fast breath (median Navax/0.9% NaCl: 2 days/2 days).

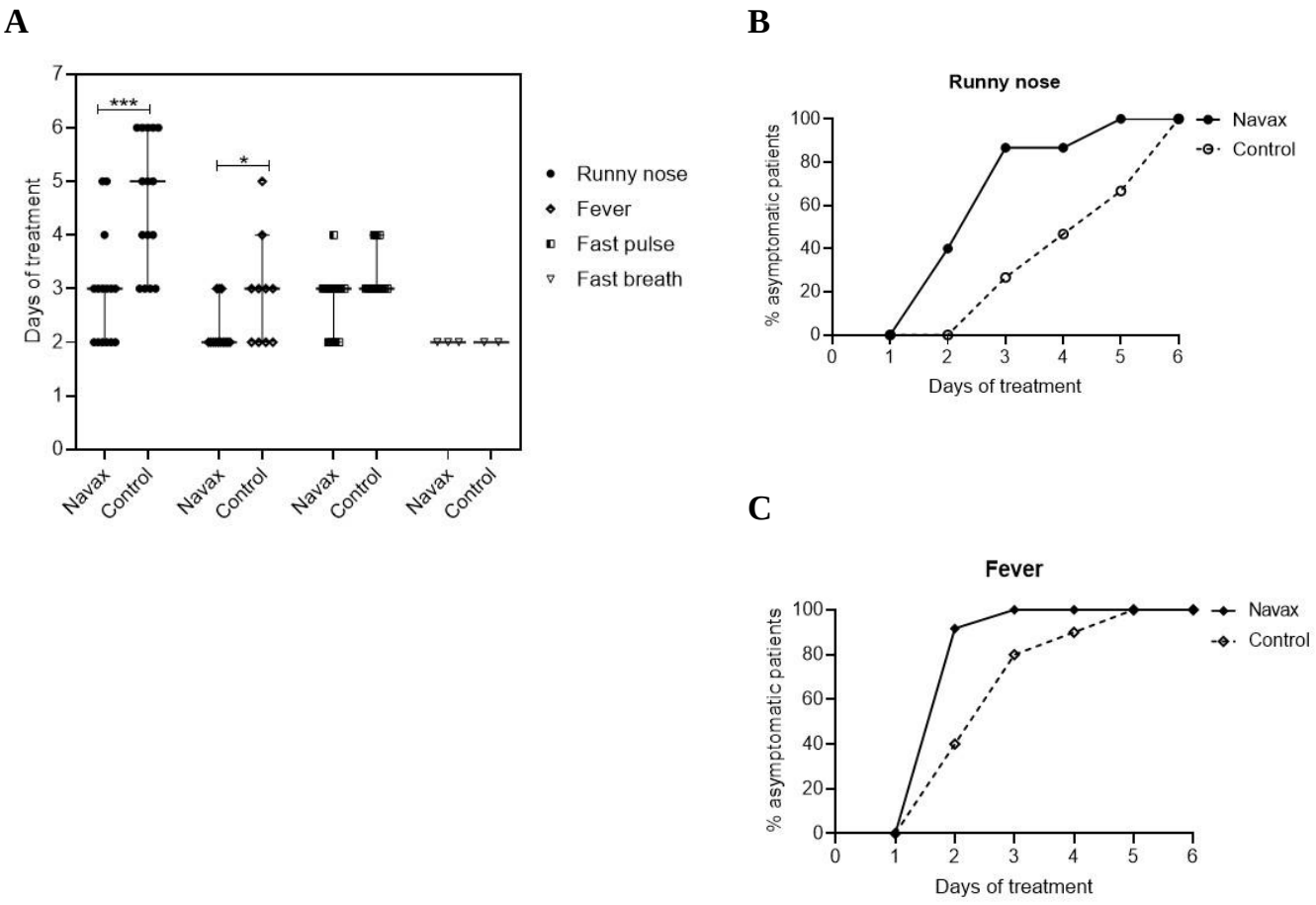

Figure 2: Recovery days of typical clinical symptoms in Navax and control group (*p<0.05; ***p<0.001)

3.3. The effect of reducing influenza viral load in nasopharyngeal samples of LiveSpo® Navax

Statistical analysis results on the value $2^{\Delta Ct}$ reflecting the reduction of influenza viral load in nasopharyngeal samples after 2 days of treatment. Compared to before the treatment on 30 patients in both groups, the group of patients using LiveSpo® Navax showed a sharp decrease in influenza viral load by about 900 folds (95% CI: 32-4421), while control groups showed a slight reduction at about 15 folds (95% CI: 2-158) (Figure 3). Therefore, LiveSpo® Navax showed impressive effect in relieving influenza viral load by about 60 folds more effectiveness than 0.9% NaCl physiological saline. The difference was statistically significant (****p<0.0001).

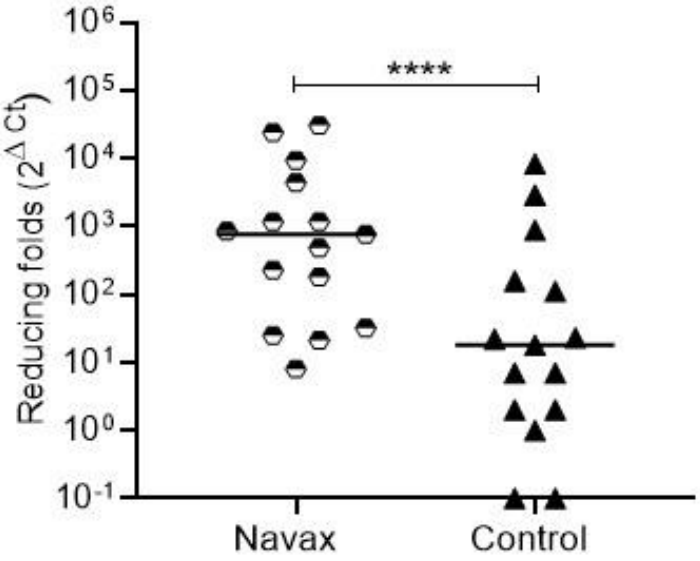

Figure 3: Reducing folds of influenza viral load after 2 days of treatment in Navax and control group (*****p*<0.0001)**

According to results on Figure 4, the *B. subtilis* and *B. clausii* spores were detected in nasopharyngeal samples of patients in Navax group at day 2. The C_t and T_m values of *B. subtilis* were about 28.0-29.6 and 85°C ± 1, respectively. The C_t and T_m values of *B. clausii* were about 31.0-35.2 and 84°C ± 1, respectively and non-detectable in control group. This result proved that patients in both groups were complied with spraying correctly with test product and placebo.

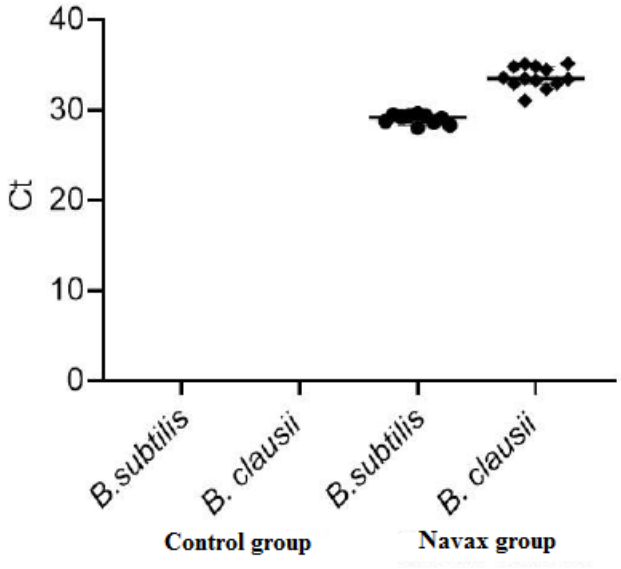

Figure 4: Threshold cycle (C_t) values of real time Real-time PCR SYBR Green for detection of spore probiotics *B. subtilis* and *B. clausii* in nasopharyngeal samples (1 mL) of patients in Navax group in comparison to control group

IV. DISCUSSION

This is the first single blind, randomized and controlled clinical trial on safety and efficacy of nasal-spraying probiotic suspension LiveSpo® Navax, which was initially performed in 30 pediatric patients with flu-positive hospitalized in International Department S, National Children's Hospital. The results on screening of participated patients showed abnormal signs of clinical symptoms and sub-clinical indexes of bronchiolitis. 100% of children were recorded with runny nose, 77% of children had fever, 80% of children had fast pulse and 16.7% had fast breath. The cardiopulmonary X-ray showed that 26/30 patients (87%) were found to have lung lesion, mainly osler's nodes or nodes combined with hyperinflation in both sides. The total white blood cells and CRP tests showed the high degree of infection, up to 57-70% of patients. Thus, the selected patients were all eligible for participated in the study. Recent studies showed that using probiotics is one of potential therapies to prevent infection of viruses and reduce the symptoms of diseases. Study of Chiba et al. (2013) was carried out in mice model expressed that using probiotic containing *Lactobacillus rhamnosus* CRL1505 orally at a concentration of 10^8 CFU/mice/day, continuously during 5 days led to relieving effect of RSV load by about 32 folds in lung at day 4 and protecting lung cells through ability to stimulate the immune system to produce IFN-γ and IL10 [16]. Another research using *Lactobacillus rhamnosus* GG represented the symptomatic-relieving and prevention effects of respiratory infections through regulation mechanism for cytokine such as TFN-α, IL-6, IL-8 [17,18]. Although the bacteria strains belong to *Lactobacillus* sp. have a long history of safe use and many scientific proofs of beneficial effect on respiratory tract, however, the probiotic products containing *Lactobacillus* ssp. do not stable with high temperature during storage, leading to survival rate reduces significantly upon the time, then the efficacy of products decreases. Moreover, these products can only use orally, so it needs time to take effectiveness and difficult to make the most of efficacy on inhibit or directly kill the disease-caused viruses or bacteria in upper respiratory tract.

Recently, our research team assessed the efficacy of LiveSpo® Navax in relieving clinical respiratory symptoms of RSV infection. The results showed that LiveSpo® Navax probiotic treatment resulted in 1-day faster recovery time than 0.9% NaCl for the following typical symptoms: runny nose, difficult breath, dry rales, moist rales, chest depression. After 3 days of treatment, RSV load in nasopharyngeal samples of patients in Navax and control group decreased at about 300 and 15 folds, respectively. Mean that LiveSpo® Navax provided 20 folds more effectiveness in reducing RSV load when compared to 0.9% NaCl physiological saline (14). To continue this research, we conducted the trial on flu-infected children and achieved similar results. Specifically, patients in Navax group had faster recovery time of runny nose (by 2 days) and fever (by 1 day) than those in control group. After 2 days of treatment, influenza viral load reduced sharply by about 900 folds in Navax group while decreased slightly by about 15 folds in control group, equivalent to 60-fold more effectiveness in reducing the concentration of influenza virus of LiveSpo® Navax in comparison to 0.9% NaCl. Hong et al. (2019) had shown that nasal-spraying with probiotic *B. subtilis* provided reducing-effect on the concentration of

influenza virus (4-5 folds) in mice through improving the anti-viral function of alveolar macrophages. However, this research has been done in mice model and has not performed in human [19]. So, what is mechanism for explanation of efficacy of LiveSpo® Navax? *In-vitro* study by confocal fluorescence microscopy technique published by Song et al. [20] was shown the ability to adsorb virus H5N1 of purified spore *B. subtilis* surface. When adsorbed, the viruses could be "inactivated" and unable to invade to the cell.

Real time PCR SYBR Green resulted the presence of *B. subtilis* and *B. clausii* in nasal mucosa of all patients using LiveSpo® Navax, which was not detected in group of patients using 0.9% NaCl. That means patients in both groups were provided correctly with test product, properly instructed and followed nasal spray procedure and these two types of spores were able to adhere to the nasal mucosa. Results also showed that product is totally safe for children under 5 years old with recommended dose as 3 times a day during continuously 6 days of treatment, in combination with monitoring and evaluating the clinical and sub-clinical symptoms. During the trial, we did not record any case with abnormal changes of breathing rate, pulse, body temperature, SpO_2, CRP or total white blood cells reflecting infection level of participated patients. 100% of patients using LiveSpo® Navax had no abnormal signs related to digestion (vomiting, diarrhea) or nasal mucosa irritation. Due to COVID-19 pandemic throughout the entire trial period, the number of hospitalized flu-infected patients at International Department S, National Children's Hospital was very small, while some of them dropped out of the study. This leads to the number of patients remaining until the end of study was only 15 patients/group, achieved 71% (15/21) of sample size as expected in research design. Therefore, the statistically significant difference on efficacy of shortening the treatment time for fast breath and fast pulse symptoms has not been evaluated. With this limited sample size, we have not been able to evaluate the effect of product on different age groups and symptom levels. However, the initial results showed that LiveSpo® Navax treatment resulted in faster recovery time and better effect in relieving typical symptoms of flu such as runny nose and fever, the difference was statistically significant. In future, clinical trials need to be expanded with larger sample sizes and added with assessment of changes in proinflammatory cytokine (IL-6, IL-8 and TFN-α) in order to more comprehensively assess the safety and efficacy of LiveSpo® Navax in relieving clinical symptoms and regulating the over-secretion of proinflammatory cytokines, as a scientific basis for the development of nasal-spraying spore probiotics product for prevention and supportive treatment of respiratory disease due to influenza virus infection.

V. CONCLUSION

This study is the first blind, randomized and controlled clinical trial for assessment of safety and efficacy of nasal-spraying liquid suspension of *Bacillus* spore probiotics in relieving clinical respiratory symptoms of flu-infected children. Despite the limited sample size, the initial results was statistically significant different between Navax group and control group, showing the effectiveness of nasal-spraying liquid suspension containing *B. subtilis* ANA4 and *B. clausii*

ANA39 spore probiotics LiveSpo® Navax is completely safe for children during 6 days of treatment, effectively shortening the time of treatment by 1 day for fever and 2 day for runny nose, reducing the influenza viral load in nasopharyngeal samples of patients by 60-fold more effectiveness than 0.9% NaCl physiological saline. This is scientific basis for expanding the sample sizes in the next clinical trial, which evaluates the efficacy of nasal-spraying spore probiotics *Bacillus* in supportive treatment of respiratory infected diseases and prevention from virus or bacterial infections.

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REFFERENCES:

1. Paules C, Subbarao K. Influenza. Lancet. 2017; 390 (10095): 697 – 708.
2. Peteranderl C, Herold S, Schmoldt C. Human Influenza Virus Infections. Semin Respir Crit Care Med. 2016; 37 (4): 487 – 500.
3. Eccles R. Understanding the symptoms of the common cold and influenza. Lancet Infect Dis. 2005; 5 (11): 718 – 725.
4. Morgan AL, Maria W, Jasmine C. Climate change and influenza: A scoping review. The Journal of Climate Change and Health. 2022; 5.
5. NBC. Health New: Influenza might be spread simply by breathing, study finds. Truy cập ngày 31/3/2022. https://www.nbcnews.com/health/health-news/influenza-might-spread-air-study-finds-n839166.
6. Angela MB, Caporuscio J, Pharm D. The flu: Is it contagious? Medical new todays, 2020. Truy cập ngày 31/3/2022. https://healthgrade.info.dyndns.tv/articles/is-the-flu-contagious.
7. Mani CS. Acute Pneumonia and Its Complications. Principles and Practice of Pediatric Infectious Diseases (Fifth Edition). 2018; 238 – 249.e4.
8. Mousa HA. Prevention and Treatment of Influenza, Influenza-Like Illness, and Common Cold by Herbal, Complementary, and Natural Therapies. J Evid Based Complementary Altern Med. 2017; 22 (1): 166 – 174.
9. CDC review 2019. Prevent Flu: Flu Vaccine Safety Information. Truy cập ngày 31/3/2022. https://www.cdc.gov/flu/prevent/general.htm.
10. Oo C, Barrett J, Hill G, et al. Pharmacokinetics and dosage recommendations for an oseltamivir oral suspension for the treatment of influenza in children. Paediatr Drugs. 2001; 3 (3): 229 - 236.

11. Kaźmierczak-Siedlecka K, Skonieczna-Żydecka K, Hupp T, et al. Next-generation probiotics - do they open new therapeutic strategies for cancer patients? Gut microbes. 2022; 14 (1): 2035659.
12. Singh TP, Natraj BH. Next-generation probiotics: a promising approach towards designing personalized medicine. Crit Rev Microbiol. 2021; 47 (4): 479 – 498.
13. Lehtoranta L, Pitkaranta A, Korpela R. Probiotics in respiratory virus infections. Eur J Clin Microbiol Infect Dis. 2014; 33: 1289 – 1302.
14. Trần Thanh Tú, Phùng Thị Bích Thủy, Bùi Thị Huyền, cộng sự. LiveSpo® Navax chứa bào tử lợi khuẩn *Bacillus* hỗ trợ hiệu quả điều trị triệu chứng và giảm nồng độ vi rút hợp bào hô hấp (RSV) ở trẻ em. Tạp chí Nhi Khoa. 2021; 14 (2): 35 - 43.
15. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008; 3 (6): 1101 – 1108.
16. Chiba E, Tomosada Y, Guadalupe MVP, et al. Immunobiotic *Lactobacillus rhamnosus* improves resistance of infant mice against respiratory syncytial virus infection. Int Immunopharmacol. 2013; 17: 373 – 382.
17. Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy-a randomised, double-blind, placebo-controlled study. Br J Nutr. 2009; 101: 1722 - 1726.
18. Harata G, He F, Hiruta N, et al. Intranasal administration of *Lactobacillus rhamnosus* GG protects mice from H1N1 influenza virus infection by regulating respiratory immune responses. Lett Appl Microbiol. 2010; 50: 597 - 602.
19. Hong JE, Kye YC, Park SM, et al. Alveolar macrophages treated with *Bacillus subtilis* spore protect mice infected with respiratory syncytial virus A2. Front Microbiol. 2019; 10: 447.
20. Song M, Hong HA, Huang JM, et al. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. Vaccine. 2012; 30: 3266 - 3277.

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