Effect of Probiotic on Rhinovirus Induced Colds

NCT 02679807

13 October 2017

Effect of Probiotic on Rhinovirus Induced Colds

Protocol number: MK EPRIC

Sponsor: Danisco Sweeteners Oy

Sponsor Address: Active Nutrition
Sokeritehtaantie 20
FI-02460 Kantvik, Finland

Protocol version number: 5.0

Date of issue: <October 13, 2017>

DECLARATIONS OF SPONSOR AND INVESTIGATORS

Declaration of sponsor

This clinical study protocol was subject to critical review and has been approved by the sponsors. The information it contains is consistent with:

- The current risk-benefit evaluation of the study product
- The moral, ethical, and scientific principles governing clinical research as set out in the Declaration of Helsinki and the principles of GCP as described in the ICH Harmonized Tripartite Guideline Topic E6: *''Guidelines for Good Clinical Practice''*, as well as in the applicable local guidelines.

The investigator will be supplied with details of any significant or new findings, including adverse events, relating to treatment with the study product.

Declaration of the investigators

I confirm that I have read the above protocol. I understand it, and I will work in accordance with the moral, ethical, and scientific principles governing clinical research as set out in the Declaration of Helsinki and GCP guidelines.

RESPONSIBLE PERSONNEL

Principal Investigator	Ronald B. Turner, MD, Department of Pediatrics P.O. Box 800386 University of Virginia School of Medicine Charlottesville, Virginia 22908, RBT2N@hscmail.mcc.virginia.edu Telephone: +1-434-243-9864, Fax: +1-434-982-4246
Study Manager / Immunoanalyses, Sponsor	Markus Lehtinen, PhD, R&D Manager, Sokeritehtaantie 20 02460 Kantvik, Finland, markus.lehtinen@danisco.com, Telephone: +358 40 1397813, Fax: +358 10 431 5555
Project Manager	Liisa Lehtoranta, PhD, Scientist Sokeritehtaantie 20 02460 Kantvik, Finland Telephone: +358 407 667 418 Fax: +358 10 431 5555 liisa.lehtoranta@dupont.com
Study Statistician	Teppo Huttunen 4Pharma, Ltd Lemminkäisenkatu 1 FI-20520 TurkuFinland Telephone: +358 40 582 3793 teppo.huttunen@4pharma.com
Database Manager	Jari Turunen 4Pharma, Ltd Lemminkäisenkatu 1 FI-20520 Turku Finland Telephone: +358 44 029 7783 jari.turunen@4pharma.com
Study Monitor	Jackie Edwards, RN, MHA, CCRA, Office: 434 985-9890 Mobile: 434 249-2564 Fax: 434 990-5772 jedwardscra@yahoo.com

PROTOCOL SYNOPSIS

Study title	**Effect of Probiotic on Rhinovirus Induced Colds**
Study phase	**II**
Study objectives	**To study the effect of probiotic supplementation on common cold illness episodes induced by rhinovirus.**
Study population	**Healthy adults, aged 18-60**
Inclusion and exclusion criteria	**INCLUSION CRITERIA AT ENROLLMENT:** • Subject must be 18-60 years of age • Subject must read and sign a copy of the approved Consent Form **INCLUSION CRITERIA AT DAY -28** • Female subjects must be using an effective birth control method **INCLUSION CRITERIA AT CHALLENGE:** • Female subjects must be using an effective birth control method • Subject must have a serum neutralizing antibody titer of less than or equal to 1:4 to rhinovirus type 39 **EXCLUSION CRITERIA AT ENROLLMENT:** **EXCLUSION CRITERIA AT DAY –28** • Antibiotic use within 3 months prior to study start • Female subjects with a positive urine pregnancy screen • History of use of probiotics in the preceding two weeks • Current cancer diagnosis or immunosuppressive therapy in the last 6 months

	• Any clinically significant abnormalities of the upper respiratory tract • Any clinically significant acute or chronic respiratory illness • Any clinically significant bleeding tendency by history • Hypertension that requires treatment with antihypertensive medications • History of angina or other clinically significant cardiac disease • Any medical condition that in the opinion of the Principal Investigator is cause for exclusion from the study • History of regular use (more than 3 days in 7) of tobacco products within the preceding two weeks • History of drug or alcohol abuse in the 6 months preceding the study **EXCLUSION CRITERIA AT CHALLENGE:** • Any upper respiratory infection or allergic rhinitis in the two weeks prior to the challenge • Any medical condition that in the opinion of the Principal Investigator is cause for exclusion from the study • Positive pregnancy screen prior to challenge • Use of any anti-inflammatory (steroids or NSAIDs) or cough/cold or allergy preparation in the two weeks prior to the challenge
Study design	**Randomized, double-blinded, placebo-controlled**
Study product	***Bifidobacterium animalis* subsp. *lactis* BI-04 as powder mixed into drink.**
Response variables and endpoints • Primary efficacy	**Primary efficacy** • Incidence of clinically defined rhinovirus-associated cold episodes

• Secondary efficacy • Exploratory endpoints	**Secondary efficacy** • Duration of illness episodes • Infection rate • Viral load • Proportion shedding the virus **Exploratory endpoints** • Blood transcriptomics • Fecal, nasal, and throat microbiota • Nasal cytokine response • Cold questionnaire comparison
Safety	AEs and SAEs will be followed throughout the trial
Statistics	**Sample size** A sample size of 95 completed subjects/arm would be expected to detect a reduction in rhinovirus-induced illness of 20% (from 60% to 40%) with 80% power with p_{α}= 0.05. **Methdology** The primary endpoint, the incidence of rhinovirus-associated illness episodes between the treatment groups will be tested with a chi-square test and logistic regression model. T-test, Mann-Whitney U test, chi-square test and ANOVA will be used for secondary parameters. Descriptive statistics for safety and other variables will be reported.

ABBREVIATIONS

ADR	Adverse Drug Reaction
AE	Adverse Event
Bl-04	*Bifidobacterium animalis* subsp. *lactis* Bl-04
eCRF	Electronic Case Report Form
CSR	Clinical Study Report
DMP	Data Management Plan
DVP	Data Validation Plan
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
GCP	Good Clinical Practice
HLA	Human Leukocyte Antigen
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
IEC	Independent Ethical Committee
IP	Investigational Product
IRB	Institutional Review Board
ITT	Intented To Treat
MedDRA	Medical Dictionary for Regulatory Activities
NSAID	Nonsteroidal anti-inflammatory drugs
PBMC	Peripheral Blood Mononuclear Cells
qPCR	Quantitative Polymerase Chain Reaction
RV	Rhinovirus
SAE	Serious Adverse Event
TMF	Trial Master File

SCHEDULE OF EVENTS

	Screening	Day -28	Day -21	Day -14	Day -7	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6-14	Day 21-28
Consent	x												
Review of inclusion/exclusion	x					x							
Serum neutralizing antibody	x*					x							x
Urine Pregnancy Screen for females		x				x							
Start of dietary run-in period	x												
Compliance check/ resupply			x	x	x	x							
Probiotic/placebo treatment		x	x	x	x	x	x	x	x	x	x	x	
Assessment of blinding						x							
Nasal swab for microbiomics		x				x			x				
Throat swab for microbiomics		x				x			x		x	x(14)	
Throat swab for culture						x					x		
Fecal sample for microbiomics						x							
Blood for transriptomics		x				x		x					
Nasal lavage for viral PCR						x							
Nasal lavage for IL-8/multiplex		x		x		x	x	x	x	x	x		
Symptom assessment						x	x	x	x	x	x		
Rhinovirus challenge						x							
Nasal lavage for quantitative RV culture							x	x	x	x	x		
Symptom diary												x	
Assessment of Adverse Events			x	x	x	x	x	x	x	x	x	x	x
Assessment of Concomitant Medications			x	x	x	x	x	x	x	x	x		x

* Volunteers will be screened for neutralizing antibody under an existing screening protocol at the University of Virginia (IRB-HSR #9948). Volunteers who are found to have antibody titers ≤1:4 to the study virus will be invited to consent for participation in the EPRIC study. Only volunteers who were tested more than 12 weeks prior to the screening visit will have serology repeated at this visit.

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1. INTRODUCTION

1.1 Background

The common cold is a ubiquitous illness of man that is associated with significant medical and socioeconomic consequences. Current treatments for the common cold that have proven efficacy are limited to pharmacologic agents that are directed at specific symptoms. These treatments- antihistamines, nasal decongestants and analgesics- have limited effectiveness, generally relieving the target symptom by 15-25% at the peak of activity, and are associated with bothersome side effects. There are no currently effective treatments for prevention of rhinovirus infections.

Most of the URTIs (i.e. common colds) (30-80 %) are caused by rhinoviruses, but also coronaviruses (10-15 %) and influenza (5-15 %) are found in significant number of URTI cases. A recent systematic review addressed the efficacy of probiotics on upper respiratory tract infections (URTI): “Probiotics were better than placebo in reducing the number of participants experiencing episodes of acute URTIs, the rate ratio of episodes of acute URTI and reducing antibiotic use.” [1]. Positive effect on enhancing influenza vaccination efficacy has been shown for probiotics in human clinical studies [2-4], but so far no studies have been published on efficacy of probiotics against rhinovirus infections.

The effect of BI-04 on the incidence of respiratory tract illness symptoms in healthy active adults was studied in a randomized, double-blind, placebo-controlled clinical trial [5]. The study was conducted in Australia by the Griffith University. A total of 229 health active adults were randomized to placebo (n = 127) and BI-04 (n = 102) groups. The probiotic was administered with a total daily dose of 2.0×10^9 CFU for 5 months throughout the primary cold and flu season. The participants reported selfassessed symptoms of respiratory illness – scratchy or sore throat, sneezing, runny or stuffy nose – using an online questionnaire. An upper respiratory tract illness (URTI) episode was recorded when two or more symptoms were present for three or more consecutive days. During the study period, subjects in the BI-04 group had 102 (59 single and 43 recurrent) URTI symptomepisodes compared to 127 (67 single 60 recurrent) episodes in the placebo group. Recurrent events analysis showed a 27% risk reduction (p=0.022) for any URTI episode in the BI-04 group compared to the placebo group. Further analyses indicated that in the BI-04 group there was a significant delay in the onset of URTI symptoms (median time 3.2 months), compared to the placebo group (median time 2.5 months) [6].

A recent study using the experimental rhinovirus challenge model at the University of Virginia (manuscript submitted) assessed the effect of BI-04 on host responses to rhinovirus infection. One-hundred fifty-two seronegative volunteers who had received BI-04 (n=73) or placebo (n=79) for 28 days were challenged with RV39. Administration of study intervention was then continued for five days during collection of specimens for assessment of host response, infection, and symptoms. Fifty-eight probiotic and 57 placebo volunteers met protocol defined criteria for analysis. Analysis of nasal lavage IL-8 concentration as the primary outcome variable revealed significantly higher concentrations of IL-8 on Day 0 prior to virus challenge in the probiotic supplemented volunteers (97 versus 58 pg/mL, respectively, p=0.25). In contrast, the IL-8 response (change in IL-8 compared to day 0 over days 1-5) to RV39 challenge was significantly reduced in the probiotic supplemented group (geometric mean ratio for change, probiotic: placebo=0.65, p=0.03). The administration of probiotic was associated with a reduction in nasal lavage virus titer and the proportion of subjects with virus

shedding was lower in the probiotic treated subjects (76% in the probiotic group, 91% in the placebo group, p=0.04). There was no effect of probiotic treatment on symptom severity, lower respiratory inflammation or serum antibody responses to the study virus. This study demonstrates that ingestion of BI-04 has a significant effect on the baseline state of innate immunity in the nasal mucosa and on the subsequent response of the host to rhinovirus challenge.

1.2 Study product

Probiotics have been defined by FAO/WHO as "Live microorganisms which when administered in adequate amount confer a health benefit on a host." The most common probiotics belong to *Lactobacillus* or *Bifidobacterium* genera. Bifidobacteria are natural human gut inhabitants that were discovered over a hundred years ago from the feces of breast-fed infants. The study product *Bifidobacterium animalis* subsp. *lactis* BI-04 (BI-04) has been genetically characterized as *B. animalis* subsp. *lactis* by 16S rRNA gene sequencing and full genome sequence comparison [7, 8]. BI-04 has been deposited in the American Type Culture Collections safe deposit as SD5219.

1.3 Summary of known potential risks and benefits to human subjects

Bifidobacterium spp. has long been considered safe and suitable for human consumption with several published studies addressing its safety [9-12]. BI-04 has been used safely in human clinical trials that have included children, adults, and elderly (Table 1). Furthermore, *Bifidobacterium lactis* has been present in human food for decades and is listed in the Inventory of Microorganisms With Documented History of Use in Human Food [13]. The European Food Safety Authority has also added the species to the Qualified Presumption of Safety list (efsa.europa.eu) and BI-04 has self-affirmed GRAS (Generally Recognized As Safe) status from FDA. Harmful or toxigenic activities have not been associated with *B. lactis* and acquired antibiotic resistance was not detected in *B. lactis* BI-04 during screening by the EU-funded PROSAFE project.

In human clinical studies, BI-04 has been safely used as a single entity and in combination with other probiotics and/or prebiotics (Table 1). The age of the subjects in these trials ranged between children 4.2 yrs and elderly 90 years. Most of the studies were conducted in healthy subjects, but some also involved subjects with allergic history. Table 2 below provides further details on these trials – number of subjects, dose and length of supplementation (single entity or combination treatment) and rate of adverse events.

In the clinical trial by West and colleagues a thorough safety and tolerability analysis was conducted for sub-group of 84 subjects (39 in BI-04 and 45 in placebo groups)[5, 14]. None of the participants in the sub-group had reported any clinically significant adverse events. Blood samples were taken before and after the 150 days of supplementation and routine haematology, and clinical chemistry markers (incl. electrolytes, liver and kidney function and metabolic markers, and C-reactive protein) were analysed. No significant changes were observed in the BI-04 group over the supplementation period and all the values were within the normal ranges.

In the Turner study (Turner et al. unpublished), AEs in 5 subjects (4 GI, 1 Respiratory illness) were judged, prior to unblinding, to be possibly or probably related to the study interventions. No serious adverse effects have been associated with the administration of BI-04.

Table 1. BI-04 supplementation in human clinical trials.

Reference	Number and characteristics of subjects consuming BI-04	Supplementation dose and length	Adverse events
Turner et al. 2015; Unpublished	95/190 Adults (Mean 22 yrs)	2 × 10^9 CFU/d for 32 d	Gastrointestinal AEs occurred only in the active group (P<0.01). AEs in 5 subjects (4 GI, 1 Respiratory illness) were judged, prior to unblinding, to be possibly or probably related to the study interventions.
[5, 6, 14]	161/465 Adults (average 36 y) Physically active	2 × 10^9 CFU/d for 150 d	2 subjects withdrew because of uncomfortable GI symptoms
[15]	336/503 Adults (30-70 y) Receiving antibiotics	4.3 × 10^9 CFU/d (n=168) or 1 × 10^9 CFU/d (n=168) in a mixture of four strains for 10 to 21 d	AE rates were 7.2%, 4.2% and 4.2% in the placebo, high-dose and low-dose groups, respectively (AEs were allergy to sea food, arrhythmia, fever, headache, left upper arm fracture, runny nose and vomiting) One SAE was reported (death due to myocardial infarction; the subject had a history of coronary heart disease) None of the AEs were considered to be related to the study product High-dose probiotic group had fewer drop-outs than low-dose or placebo group
[16]	24 / 47 Children (mean 9 yrs) with history of allergy	1.25 × 10^9 CFU/d with one other strain for 122 d	2 subjects dropped out in probiotic group, no reason published
[17]	20/40 Adults (18-45y) Consuming antibiotics	1 × 10^{10} CFU/d in a mixture of five strains for 20 d	No significant difference in AE rate between treatment groups (both groups consumed antibiotics) Most common AEs were diarrhea (3 in active and 1 in placebo group), vaginal yeast infection (4 in active and 1 in placebo group) and abdominal pain (3 in probiotic and 3 in active group)
[18]	9/86 Adults (18-64 y) Oral cholera vaccine given	2 × 10^{10} CFU/d for 3 w	None documented
[19]	20/40 Adults (>18y) Consuming antibiotics	5 × 10^9 CFU/d in a mixture of five strains for 20 d	None documented
[7]	9/18 Healthy elderly	3.5 × 10^{10} CFU/d in a mixture with inulin and Bb-02 for 28 days	None documented

2 STUDY OBJECTIVES

2.1 Purpose of the trial

To test the hypothesis that prophylaxis of human volunteers with probiotic *Bifidobacterium animalis* subsp. *lactis* BI-04 will reduce the incidence of rhinovirus induced cold episodes.

2.2 Objectives

2.2.1 Primary objective

- To study the effect of probiotic supplementation on incidence of rhinovirus-associated common cold illness episodes.

2.2.2 Secondary objectives

- To study the effect of probiotic supplementation on the infection rate and duration of illness episodes
- To examine the effect of the probiotic on the rhinovirus load in the nose

2.2.3 Exploratory objectives

- To study the effect of the probiotic on blood transcriptome during basal state and infection.
- To validate the effect of the probiotic on nasal cytokine profile at baseline and during infection
- To characterize the nasal, throat, and intestinal microbiomes before and during rhinovirus infection and supplementation period.
- To investigate correlations between specific microbiome profiles, immune responses and subsequent infection and illness.
- To compare with a cold questionnaire

3 STUDY POPULATION

Approximately 900 healthy, adult (18-60 years) volunteers will be recruited for participation in these studies via a screening protocol (IRB #9948). All volunteers will be tested for the presence of serum neutralizing antibody to rhinovirus type 39. Volunteer subjects with an antibody titer ≤1:4 will be eligible for participation.

3.1 Inclusion Criteria

INCLUSION CRITERIA AT ENROLLMENT:

- Subject must be 18-60 years of age.
- Subject must read and sign a copy of the approved Consent Form
- Subject must have a serum neutralizing antibody titer of less than or equal to 1:4 to rhinovirus type 39

INCLUSION CRITERIA AT DAY -28

- Female subjects must be using an effective birth control method.

INCLUSION CRITERIA AT CHALLENGE:

- Female subjects must be using an effective birth control method.

3.2 Exclusion Criteria

EXCLUSION CRITERIA AT ENROLLMENT:

EXCLUSION CRITERIA AT DAY -28

- Antibiotic use within 3 months prior to day -28
- Female subjects with a positive urine pregnancy screen
- History of use of probiotics in the preceding two weeks
- Current cancer diagnosis or immunosuppressive therapy in the last 6 months
- Any clinically significant abnormalities of the upper respiratory tract
- Any clinically significant acute or chronic respiratory illness
- Any clinically significant bleeding tendency by history
- Hypertension that requires treatment with antihypertensive medications
- History of angina or other clinically significant cardiac disease
- Any medical condition that in the opinion of the Principal Investigator is cause for exclusion from the study
- History of regular use (more than 3 days in 7) of tobacco products within the preceding two weeks
- History of drug or alcohol abuse in the 6 months preceding the study

EXCLUSION CRITERIA AT CHALLENGE:

- Any upper respiratory infection or allergic rhinitis in the two weeks prior to the challenge
- Female subjects with positive pregnancy screen prior to challenge
- Any medical condition that in the opinion of the Principal Investigator is cause for exclusion from the study
- Use of any anti-inflammatory (steroids or NSAIDs) or cough/cold or allergy preparation in the two weeks prior to the challenge

3.3 Recruitment, screening, and randomization

Approximately 900 healthy, young adult (18-60 years) volunteers will be recruited for participation in these studies by posted, newspaper, or radio advertisements. Volunteers will be tested for the presence of serum neutralizing antibody to rhinovirus type 39 under an existing screening protocol at the University of Virginia (IRB-HSR #9948). Volunteer subjects with an antibody titer ≤1:4 will be invited to consent for participation in this (EPRIC) study. Volunteers who were screened for antibody more than 8 weeks prior to consent for the EPRIC study will have a repeat serology done at the screening visit to assure eligibility.

Volunteers enrolled in the study will be randomized 1:1 to the study product or placebo. The sponsor will supply the study product and a matching placebo. The randomization schedule will be provided by the sponsor. The blinding code will be held in confidence by the School of Medicine Clinical Trials Office at the University of Virginia.

3.4 Subject Withdrawal and replacement of subjects

3.4.1 Withdrawal criteria

Subjects may be withdrawn from the study (i.e. from any further study product or study procedure but not from analyses) for the following reasons:

- At their own request or at the request of their legally authorized representative.
- If, in the investigator's opinion, continuation in the study would be detrimental to the subject's well-being.
- If they are lost to follow-up.

In all cases, the reason for and date of withdrawal must be recorded in the eCRF and the sponsor's representative must be notified within 5 days. The subject must be followed up to establish whether the reason was an adverse event, and, if so, this must be reported.

The investigator must make every effort to contact subject lost to follow-up. Attempts to contact such subject must be documented in the subject's records (e.g., dates and times of attempted telephone contact).

Any subject may be withdrawn from the study at the discretion of the investigator. The subject is also free to terminate his participation at any time. An end of study visit will then be organized.

3.4.2 Withdrawn subject data collection

The principal investigator and/or other investigator involved in the study will document on the termination page of the eCRF the reason for the subject withdrawal as follows:

- Lost to follow-up: subjects who leave the study unnotified or do not attend the study visit or cannot be contacted by phone. Intensive efforts should be made to locate and recall them if possible and to determine their health status at a minimum.
- Adverse event: an adverse event form must be completed.
- Adverse laboratory event: this reason should be stated when a laboratory value is interpreted as a clinically significant abnormal value not explained by a laboratory error or being not a known or abnormal value commonly observed in this type of population.
- Deviation from protocol.
- Consent withdrawn.
- Other: if no above mentioned reasons are applicable, then the reason will be specified.

3.4.3 Replacement of subjects

Subjects withdrawn from the study will not be replaced.

4 Experimental Design

4.1 Overall Study Design

This double-blinded, randomized, controlled study will be conducted at the University of Virginia. The study will use the experimental rhinovirus human challenge model.

4.1.1 Number of subjects

Approximately 900 volunteers will be recruited for participation in these studies. Volunteers seronegative to rhinovirus type 39 will be randomly treated for 42 days with either:

- probiotic (n=130)
- placebo (n=130)

4.1.2 Study center

The study will take place at the University of Virginia, Charlottesville.

4.2 Assessments and Procedures

4.2.1 Study Conduct

The study will be done in five cohorts of 60-70 subjects each for logistical reasons. The first cohort will be conducted in the Spring of 2016. Subsequent cohorts will be done in Fall 2016, Spring 2017, Fall 2017 and Spring 2018. The assays and data collection for analysis of the primary and secondary efficacy endpoints will be substantially complete by December, 2018.

4.2.2 Study periods

Screening:

- Consent and enrollment (approximately 375 subjects)
- Medical history questionnaire and demographics
- Vital signs and brief physical exam
- Physical activity question
- Review of inclusion/exclusion criteria
- Blood draw for screening neutralization antibody titer (if no antibody screening test has been done in the last 12 weeks)

Day -42 ± 4:

- All subjects begin dietary “run-in” period

Day -28 ± 4: Seronegative subjects only

- Review of inclusion/exclusion criteria
- Pre-probiotic baseline nasal lavage for IL-8 and other cytokines
- Pre-probiotic baseline nasal swab for microbiomics
- Pre-probiotic baseline throat swab for microbiomics
- Blood draw for pre-probiotic transcriptomics studies
- Assessment of concomitant medications
- Randomized to active or placebo probiotic
- Urine pregnancy screen for females

Day -21 ± 2

- All subjects return to study site for new supply of probiotic and compliance review
- AE assessment
- Assessment of concomitant medications

Day -14 ± 2:
- All subjects return to study site for new supply of probiotic and compliance review
- Nasal lavage for multiplex cytokine assays
- AE assessment
- Assessment of concomitant medications

Day -7 ± 2:
- All subjects return to study site for new supply of probiotic and compliance review
- AE assessment
- Assessment of concomitant medications

Day 0:
- Review of inclusion/exclusion criteria
- Assessment of blinding
- AE assessment
- Assessment of concomitant medications
- Urine pregnancy screen for females
- Stool specimen for probiotic compliance and microbiomics
- Nasal swab for microbiomics
- Throat swab for microbiomics
- Throat swab for culture
- Nasal lavage for unanticipated viruses
- Nasal lavage for pre-RV challenge baseline IL-8
- Blood draw for measurement of neutralizing antibody
- Blood draw for pre-RV challenge transcriptomics
- Nasal lavage for multiplex cytokine assays
- Symptom assessment
- Challenge with rhinovirus
- Compliance check/resupply
- Continue probiotic treatment

Day 1-4:
- AE assessment
- Assessment of concomitant medications
- Nasal lavage for quantitative rhinovirus culture
- Nasal lavage for IL-8 measurement
- Nasal lavage for multiplex cytokine assays
- Symptom assessment
- Assessment of the effect of symptoms on physical activity
- Nasal swab for microbiomics (day 3)
- Throat swab for microbiomics (day 3)
- Blood draw for post-RV challenge transcriptomics (Day 2 only)
- Continue probiotic treatment

Day 5:
- AE assessment
- Assessment of concomitant medications
- Nasal lavage for quantitative rhinovirus culture
- Nasal lavage for IL-8 measurement
- Throat swab for culture
- Throat swab for microbiomics
- Symptom assessment
- Assessment of the effect of symptoms on physical activity
- Assessment of common cold illness
- Compliance check/resupply
- Continue probiotic treatment

Day 14
- Return symptom diaries and any remaining study product
- Discontinue probiotic treatment
- Throat swab for microbiomics

Day 21-30:
- AE assessment
- Assessment of concomitant medications
- Blood draw for serum neutralizing antibody

4.2.3 Pre-study safety assessment (Screening)
Volunteers who agree to participate will have a brief medical history and brief physical assessment prior to participation in the study. The purpose of these evaluations will be to detect any underlying condition that would either place the subject at increased risk from the study procedures or that would compromise evaluation of the study endpoints. A medical history questionnaire will be completed by all study subjects. The completed questionnaire will be reviewed with study personnel and all positive responses clarified. A brief physical examination consisting of pulse, respiratory rate, body temperature, blood pressure, and visual examination of the nose will be performed on all subjects and all female subjects will have a urine pregnancy screen. The urine pregnancy screen will be repeated on Day 0 prior to virus challenge. Subjects will be asked whether they exercise at least 30 minutes 3 times each week over the last 3 months. All subjects must meet the inclusion criteria and must not meet any of the exclusion criteria for participation in the study.

4.2.4 Nasal lavage (Study Day -28, -14, and 0-5)
Nasal lavage will be collected for virology and for determination of IL-8 and other cytokine concentrations. These specimens are collected by instillation of 5 mL of sterile 0.9% saline into each nostril. This wash is then immediately expelled into a waxed paper cup and kept chilled until processed.

4.2.5 Blood for transcriptomics (Study Days -28, 0, and 2)
Blood specimens will be collected into PaxgeneRNA extractor tubes for subsequent analysis of gene expression by microarray. These specimens will be handled according to the manufacturer's instructions and stored at -70C until they are analyzed.

4.2.6 Nasal and throat swab sample collection (Study Day -28, and 0, 2, 3,5, 14)
Nasal swabs will be collected and stored at -70C. Nasal swabs will be collected by inserting a moistened nylon flocked swab (FloQswabs, Copan Diagnostics Inc, Murrieta, CA) into each naris until resistance is met. The swab will be gently rotated and then withdrawn. Pharyngeal swabs will be done using the same swab material to swab the posterior pharyngeal wall. The nasal swabs and throat swabs will be collected before the daily dose of the study product. All throat swabs will be collected by a study nurse experienced in the procedure.

4.2.7 Assessment of Compliance (Study Days -21,-14,-7, and 0-14)
At each study visit during the pre-challenge dosing period, volunteers will be asked to return unused study product to the study site. Compliance will be assessed by counting missed doses of study product. For purposes of the per protocol analyses, the subject will be assessed as compliant with the protocol if at least 80% of the planned doses were taken. In addition, DuPont Nutrition and Health will measure the amount of *Bifidobacterium animalis* subsp. *lactis* from the fecal samples using qPCR.

4.2.8 Stool sample collection (Study Day 0)
Fecal samples will be collected into an air tight container and frozen. The subjects will be given stool sample containers, outer packaging for hygienic storage and delivery and written instructions including sampling and clinic visit dates at a visit prior to sample collection. A prior notice as a reminder will be sent to all subjects before the sampling and clinic visit day. All samples will be marked with study subject code and date.

The stool samples should be:

- defecated on the same day
- kept in a refrigerator or on ice before transport to the clinic
- immediately frozen to -70°C at the clinic
- kept frozen at all times until analysis

4.2.9 Assessment of blinding (Study Day 0)
Volunteers will be asked whether they believe they are receiving active or placebo product. On study day 0, before challenge with the study virus, each volunteer will indicate their response to the following question:

Which treatment do you think you are taking: active control don't know

4.2.10 Challenge with rhinovirus (Study Day 0)
The challenge virus to be used in this study is rhinovirus, type 39, a serotype that attaches via the ICAM-1 receptor. Inoculation will be with a challenge pool of virus that has been thoroughly safety tested and approved for use for virus challenge experiments by the FDA (IND #12934). Following a nasal wash, each subject will be given the challenge virus by intranasal drops. The virus is administered in a volume of 0.25 mL/nostril with a calibrated pipette and two inoculations of virus are given several minutes apart. The total virus inoculum will be 20-100 $TCID_{50}$/volunteer contained in 1 mL of fluid. This procedure is used routinely in the rhinovirus challenge model and results in an infection rate of 85-90% in susceptible volunteers.

4.2.11 Symptom scoring (Study Days 0-14)
Symptom scores on study days 0-5 will be collected in an interactive interview with the study staff. Symptoms will be assessed using the modified Jackson criteria. On study days 6-14, volunteers will be asked to complete a symptom diary that will consist of the same symptoms assessed by the Jackson criteria. Volunteers will also be asked whether their symptoms affected their physical activity.

5 Study Product

5.1 Study Product and Comparator

5.1.1 Investigational product
The study product will be a $2*10^9$ cfus of probiotic *Bifidobacterium lactis* Bl-04 (DuPont Nutrition and Health) mixed with 1g of sucrose as a carrier. The study product will be manufactured according to GMP and QC guidelines approved by FDA at DuPont production site located at Madison Wisconsin, USA.

5.1.2 Placebo
Placebo will be 1g of sucrose that has identical appearance, smell, and taste with the study product.

5.2 Supply, Packaging, Labeling, Handling and Storage

Investigational product and placebo product will be released by DuPont Nutrition and Health Qualified Person (QP) and then sent to Ronald Turner, MD, Barringer 4441, University of Virginia School of Medicine, Charlottesville, VA, 22908. Study products will be packaged and labeled by number for each individual kit according to the study plan. The individual dose boxes (i.e. kits) will be labeled to include the following information:

- Sponsor study code
- Sponsor's name and address
- Investigational product (name) powder and/or corresponding placebo
- Amount of powder etc. of X g/mg and/or corresponding placebo
- For oral administration only
- Instruction of intake
- Investigator's name and study site
- Kit and Batch number
- Expiry date
- Storage conditions
- For clinical trial use only. Keep out of reach of children.

5.2.1 Study product supply and storage
Study product and placebo will be produced under GMP conditions and will be packaged ready for distribution to the volunteers. The investigational product must be stored in the refrigerator until use (2-8 °C). Sponsor may control the stability of the investigational product by collecting samples from the returned containers after the study end to check the viability of the probiotic bacteria. Study product will be stored in a locked, limited access room at +4 °C under the supervision of the study coordinator.

5.3 Dosage and Administration

5.3.1 Duration of Treament

All volunteers will sign consent and be screened for study eligibility before randomization to study product. On day –28 of the study, all volunteers will come to the study site, be randomized to study treatment and instructed when to take the first dose of study product. Volunteers will be instructed to take daily one sachet of the study product mixed into a drink that is not hot and does not contain alcohol. The study product will have a minimal of $2*10^9$ cfus of BI-04. On days -21, -14 and -7 subjects will come to the study site for re-supply of the probiotic and for evaluation of compliance. On day 0 the volunteers will be challenged with the study virus. After the virus challenge all volunteers will continue the study treatment on through study day 14.

5.3.2 Methods of Assigning Subjects to Treament

The study product and placebo will be randomized and assigned sequential numbers by the sponsor. Volunteers will be assigned a study subject ID number as they present for the day -28 visit. The randomization number and the number of the study material they are given will correspond to their study number. All study personnel will remain blinded to the product or placebo assignment until all data have been collected and entered into the study database, and the database has been locked.

5.4 Concomitant medications

Volunteers who participate in this study will be asked to refrain from using common cold or allergy treatments for the duration of the study. If in the opinion of the volunteer their symptoms are severe and require treatment, treatment should be limited to acetaminophen and the use of the medication should be reported to the study staff. The use of medications other than common cold treatments will be permitted at the discretion of the investigator. All volunteers will be asked to refrain from the use of NSAIDs or steroids beginning two weeks prior to the virus challenge until the end of the symptom reporting period (study day 14).

Use of antibiotics may have an impact on the investigational product functionality and therefore antibiotic use should be avoided unless explicitly needed as judged by a physician. Volunteers who take antibiotics during the study will be removed from the study.

5.4.1 Concomitant diet

During the dietary intervention period (days -28 to 14) subjects should not use any other probiotic or prebiotic containing products. A list of avoidable and acceptable yoghurt products will be provided to volunteers. Study subjects are advised not to mix the product or placebo with hot or alcohol containing drinks.

5.4.2 Prior medications

Continued use of topical agents, hormonal contraception or hormone replacement therapy, SSRIs, and other anti-depressants will generally be permitted.

5.5 Study Product Accountability

The investigator is responsible for the accounting for all unused and all used investigational product containers. The study eCRFs must have an accurate dispensing/returning log. The third-party dispenser (can be a hospital pharmacy or a study nurse) uses this information to

maintain an accurate and complete dispensing and inventory record. The designated copies of the completed dispensing and inventory record will be returned to the sponsor.
Supplies are shipped to the investigative site as needed. Study product accounting will be reviewed by the site monitor during routine monitoring visits. At the completion or termination of the study, a final study product accountability review and reconciliation must be completed, and any discrepancies must be investigated and their resolution documented. All full, partially full, and empty containers of study products must be returned to the sponsor with the appropriate form. The sponsor then destroys all and documents the destruction.

6 Response Variables and Study Endpoints

6.1 Assessment of Efficacy

6.2 Primary endpoint

6.2.1 Incidence of rhinovirus-associated illness episodes

6.2.1.1 Rhinovirus infection: Rhinovirus infection will be defined as the isolation of the challenge virus on any of the 5 days (study days 1-5) after virus challenge or a four-fold rise in neutralizing antibody titer to the study virus.

6.2.1.2 Symptomatic illness: Symptomatic illness will be defined according to the modified Jackson criteria. Volunteers who have a total symptom score of at least 6 over the 5 days (study days 1-5) after virus challenge and either three days of rhinorrhea or the subjective impression that they have had a common cold illness will be considered to have a symptomatic illness.

6.2.1.3 Rhinovirus-associated illness episodes: Volunteers who have both a rhinovirus infection and a symptomatic illness will be defined as having a rhinovirus-associated common cold illness.

6.3 Secondary endpoints

6.3.1 Duration of illness

6.3.1.1 Duration of illness will be defined as the time between the virus challenge and the end of the illness episode in volunteers who meet the Jackson definition of symptomatic illness. The end of the illness will be defined as the first of two consecutive days with a total symptom score ≤ 1 that occurs after the subject has met symptom criteria for a Jackson cold.

6.3.2 Incidence of infection

6.3.2.1 Rhinovirus infection will be defined as the isolation of the challenge virus on any of the 5 days (study days 1-5) after virus challenge or a four-fold rise in neutralizing antibody titer to the study virus.

6.3.3 Viral load during infection

6.3.3.1 Viral load will be assessed by comparing the quantitative virus titer in nasal lavage between the probiotic and placebo groups. For purposes of this calculation subjects with no virus isolate on a study day will be defined as having a quantitative titer of -0.5 ($\log_{10}$).

6.3.4 Proportion shedding the virus

6.3.4.1 The proportion of volunteers shedding virus in the placebo group will be compared with the proportion in the probiotic group. Virus shedding will be defined as the

isolation of the challenge virus on any of the 5 days (study days 1-5) after virus challenge

6.4 Exploratory endpoints

6.4.1 Cytokine response of nasal mucosa

6.4.1.1 Interleukin-8 assay: Concentrations of interleukin-8 in nasal lavage are determined with a commercially available ELISA assay (R&D Systems, Minneapolis, MN). The lower limit of detection for the assay is 31.25 pg/mL. The precision of the assay for measurement of IL-8 in nasal wash has been determined with pools of nasal wash spiked with known concentrations of IL-8. Intra-assay variability was found to range from 3.8-5.7% and inter-assay variability was found to range from 2.7-11% for nasal wash containing high and low concentrations of IL-8, respectively. The recovery of IL-8 in nasal wash ranged from 88-136% of predicted. Specimens that have IL-8 concentrations greater than the operating range of the assay will be diluted 1:10 and 1:100 and re-assayed.

6.4.2 Multiplex assays: A panel of cytokines and chemokines (TGF-β1, GCSF, GMCSF, IFN-γ, IL-1α, IL-12p70, IL-15, CCL20, IL-1β, IL-6, IL-10, CXCL10, CCL2, CCL3, CCL5, TNF-α) may be measured in nasal lavage fluid using a commercially available multiplex assay (Aushon BioSystems, Inc., Billerica, MA). A determination of which assays will be included will be made as a post-hoc exploratory analysis.Blood transcriptomics

6.4.2.1 Collection of specimens: The effect of probiotics on gene expression before and after rhinovirus challenge will be determined by comparing expression in the probiotic and placebo groups on study days -28, 0 and 2. Expression analysis will be done on peripheral blood specimens by microarrays targeted at inflammatory and antiviral genes. Blood samples will be collected in PAXgene RNA tubes (PreAnalytix) and stored at -80°C until shipping. Samples will be shipped on dry ice to DuPont Haskell Global Centers for Health and Environmental Science and immediately stored at -80°C until further processing. RNA will be isolated from blood stored in PAXgene tubes using a PAXgene Blood RNA isolation kit (Qiagen) and potential DNA contamination will be removed with on-column DNase treatment. RNA concentration and quality will be verified on a Nanodrop© spectrophotometer (NanoDrop) or with a Quant-iT™ RiboGreen® RNA Assay Kit (Thermo Fisher). RNA integrity will be determined with an Agilent Bioanalyzer 2100 (Agilent). All RNA samples processed for RNA-sequencing should have a 260/280 absorption ratio greater than 2 and a RNA integrity number (RIN) greater than 9.8.

6.4.2.2 RNA-seq: Targeted RNA-seq library preparation will be conducted with Tempo-Seq (BioSpyder) detector oligos designed against a ~1500 human gene panel focused on innate immunity and antiviral defense genes. Library preparation will be conducted in 96 sample batches. Up to 2 library preparations (n=192 samples) will be pooled, purified, quantitated and stored frozen prior to RNA-sequencing. The pooled libraries will be sent to the Pioneer core genomics facility in Johnston, Iowa for RNA-seq using an Illumina NextSeq, or HiSeq platform. The target read-depth per sample will be 1.5 million reads to ensure 500-1000 reads per gene. The reads will be quality filtered, trimmed and demultiplexed by the technical staff in Johnston. The resulting FASTQ files will be transferred via external hard drive, or through the DuPont network for further processing. Backup FASTQ files will be archived at the Johnston facility. The FASTQ files will be aligned to an annotated reference to determine the number of reads per gene. The reads will be quantitated by

normalizing with control genes in the samples set. Other quantitation methods may be considered as well. Data will be stored in this format until the samples can be decoded for further analysis. Once decoded, metadata will be added to the data files to add additional annotation to the sample identifiers and group samples according to experimental conditions. Differential expression analysis will be conducted to determine transcripts that are up or down regulated between the experimental conditions.

6.4.3 Rhinovirus type-specific antibody response

6.4.3.1 The effect of probiotic on host antibody responses to the challenge virus will be determined by comparing the convalescent neutralizing antibody titer in the probiotic and placebo groups.

6.4.4 Fecal, throat and nasal microbiota

6.4.4.1 Bacterial DNA will be isolated from the swabs and feces by using MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Bridgewater, NJ). Human DNA may be quantified for analysing the ratio of human and bacterial DNA by using housekeeping genes that do not allow subject identification. The composition of microbiota will be analyzed by next-generation sequencing (or equivalent) technology for identification of microbial species in the samples. The data will be analyzed to determine effects of probiotic on both abundance and diversity of bacterial species. . In addition, single strain targeting PCR-based molecular methods (i.e. qPCR, digital PCR or equivalent) will be applied to detect the supplemented probiotic strain, BI-04.

6.4.4.2 Throat swabs will be collected on study days 0, 5, and 14 for detection of pharyngeal pathogens by culture and qPCR. A single swab will be sent to the microbiology laboratory at the University of Virginia for detection of *S. aureus*, pneumococcus, *H. influenza*, meningococcus and group A streptococcus.

6.4.5 Cold questionnaire comparison

Performance of the questionnaire used in the West study [5] will be compared to endpoints of this study.

6.5 Assessment of safety

6.5.1 Adverse events

6.5.1.1 The safety of the study interventions will be assessed by monitoring of adverse events. Volunteers will be asked at each study visit to report any unexpected events whether or not they are apparently study related. Each event will be recorded and an assessment of relateness to the study interventions will be made by the investigator. Adverse events will be coded according to established standards (MedDRA). The analysis will compare both total and related events in the probiotic and the control group.

7 Adverse Events

7.1 Adverse event

7.1.1 Definitions

7.1.1.1 An adverse event (AE) is any untoward, undesired, or unplanned event in the form of signs, symptoms, disease, or laboratory or physiologic observations occurring in a

person given an IP in a clinical study. The event does not need to be causally related to the IP or the clinical study. An AE includes, but is not limited to the following:

- Any clinically significant worsening of a pre-existing condition.
- An AE occurring from overdose of an IP, whether accidental or intentional. Overdose is a dose greater than specified in the protocol.
- An AE occurring from abuse (e.g., use for non-clinical reasons) of an IP.
- An AE that has been associated with the discontinuation of the use of an IP.

7.1.1.2 Treatment Emergent Adverse Event and Repetition of Adverse Event: An adverse event will be regarded as treatment-emergent if it occurred for the first time after the study product administration or if it was present before, but worsened after the study product administration.

7.1.2 Assessment of Adverse Events

For the purposes of this study using experimental rhinovirus infection, physical signs and symptoms that are expected to be associated with the common cold should not be reported as adverse events. The following physical signs and symptoms are expected to be associated with the common cold: nasal obstruction (nasal stuffiness), coryza (runny nose), sore throat, cough, sneezing, myalgia, headache, feverishness, chills, loss of appetite and malaise.

Information regarding common cold-related complications must be documented appropriately. Common cold-related complications include bronchitis, bronchospasm or exacerbation of asthma, sinusitis and otitis media. For common cold-related complications, the following information should be documented: onset date, severity, seriousness, relationship to study therapy, action taken regarding study therapy, outcome, and therapy given for event.

Some subjects may experience gut discomfort during the study product consumption. The symptoms that have been associated with use of bifidobacteria include bloating, flatulence and abdominal pain. The subjects should contact the study staff in the case that symptoms persist.

Assessment of all adverse events will include recording the time of onset and resolution of the event, severity, intervention, and an attribution by the investigator of whether the event was related to the study product.

7.1.3 Halting rules for adverse events

Should any of the following occur, the entire study will be stopped

- B1-04 bacteremia or invasive infection
- Any single serious and unexpected adverse event for which attribution is assessed as possibly, probably, or definitely related to the study products.
- 10% of participants develop any severe (grade 3) gastrointestinal AE
- 10% of participants develop any severe (grade 3) non-gastrointestinal AEs of the same type.

7.1.4 Monitoring of adverse events

All volunteers will be specifically asked about any unexpected medical events at each study visit after the study treatment is begun. All volunteers will be instructed to contact the study site if they experience any unexpected symptoms during the time they are consuming the study product. At the final study visit, volunteers will be asked if they have had any unusual symptoms or problems during the study. All positive responses will be recorded. Medically significant responses will be followed until the symptom/problem is resolved or judged unrelated to the study and the subject has been referred for appropriate care.

7.2 Serious adverse events and unexpected

7.2.1 Definitions

7.2.1.1 General definitions:

A Serious Adverse Event (SAE) or Serious Adverse Drug Reaction (ADR) is any untoward medical occurrence that at any dose:

- Results in death.
- Is life-threatening (see below).
- Requires inpatient hospitalization or prolongation of an existing hospitalization (see below).
- Results in a persistent or significant disability or incapacity (see below).
- Results in cancer.
- Is a congenital anomaly or birth defect.

Additionally, important medical events that may not result in death, be life-threatening, or require hospitalization may be considered SAEs when, based on appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such events include allergic broncho-spasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in hospitalization, or development of drug dependency or drug abuse.

Life-threatening refers to immediate risk of death as the event occurred per the reporter. A life-threatening experience does not include an experience, had it occurred in a more severe form, might have caused death, but as it actually occurred, did not create an immediate risk of death. For example, hepatitis that resolved without evidence of hepatic failure would not be considered life-threatening, even though hepatitis of a more severe nature can be fatal. Similarly, an allergic reaction resulting in angioedema of the face would not be life-threatening, even though angioedema of the larynx, allergic bronchospasm, or anaphylaxis can be fatal.

Hospitalization is official admission to a hospital. Hospitalization or prolongation of a hospitalization constitutes criteria for an AE to be serious; however, it is not in itself considered a serious adverse event (SAE). In absence of an AE, a hospitalization or

prolongation of a hospitalization should not be reported as an SAE. This is the case in the following situations:

- The hospitalization or prolongation of hospitalization is needed for a procedure required by the protocol.
- The hospitalization or prolongation of hospitalization is part of a routine procedure followed by the centre (eg, stent removal after surgery). This should be recorded in the study file.

In addition, hospitalization for a pre-existing condition that has not worsened does not constitute an SAE.

7.2.1.2 **Disability** is defined as a substantial disruption in a person's ability to conduct normal life functions. If there is any doubt about whether the information constitutes a SAE, the information is treated as a SAE.

7.2.1.3 **Other Reportable Information.** Certain information, although not considered an SAE, must be recorded, reported, and followed up as indicated for an SAE. This includes:

- Overdose of an investigational product as specified in this protocol with or without an AE.
- Inadvertent or accidental exposure with or without an AE.

7.2.1.4 **Definition of expected SAE**: No type of expected SAE was defined for the purpose of this study; all SAE will therefore be considered unexpected.

7.2.2 Severity

The severity of adverse events will be graded on a three–point scale (mild, moderate, and severe) and reported in detail as indicated on the eCRF. A description of scales can be found below.

- Mild: discomfort noticed but no disruption of normal daily activity.
- Moderate: discomfort sufficient to reduce or affect daily activity.
- Severe: inability to work or perform normal daily activity.

7.2.3 Causal relationship with trial medication

The causal relationship will be rated as follows:

- Definitely not related
- Probably not related
- Possibly related.
- Probably related.
- Definitely related.

7.2.4 Reporting

Any serious adverse event occurring in a subject after providing informed consent until end of trial must be reported. Any adverse event should be recorded on the appropriate eCRF.

Unexpected serious adverse events that are fatal or life-threatening must be filed as soon as possible and the sponsor will ensure all required information are edited in a initial report submitted to the Ethics Committee as soon as possible but not later than 7 calendar days after first knowledge, followed by as complete a report as possible within 8 additional calendar days.

Unexpected serious adverse events that are not fatal or life-threatening must be filed as soon as possible but no later than 15 calendar days after first knowledge by the sponsor.

Sponsor will be notified within 24h of awareness of any SAE, and Sponsor will be informed of the situation and its development on a running basis.

8 STATISTICAL METHODS

8.1 ESTIMATION OF SAMPLE SIZE

The sample size for this study was calculated on the primary endpoint of rhinovirus-induced illnesses. The challenge model results in an infection rate of approximately 90% in the control group. Approximately 67% of the infected volunteers (60% of all challenged volunteers are expected to develop a symptomatic illness and will meet the study definition of a rhinovirus-induced illness. A sample size of 95 completed subjects/arm would be expected to detect a reduction in rhinovirus-induced illness of 20% (from 60% to 40%) with 80% power with p_{α}= 0.05.

8.2 RANDOMISATION

The study product and placebo will be randomized and assigned sequential numbers by the sponsor. Volunteers will be assigned a study number as they present for the day -28 visit and the number of the study material they are given will correspond to their study number. All study personnel will remain blinded to the product or placebo assignment until all data have been collected and entered into the study database.

8.3 BLINDING AND CODE BREAKING INSTRUCTIONS

8.3.1 Blinding

The study product and a placebo that is identical in appearance, taste and smell will be provided by the sponsor. The study material will be identified only by a number. All study personnel will remain blinded to the study material administered until all data have been collected and entered into the study database.

8.3.2 Emergency unblinding of a individual subject

The randomization schedule will be provided by the sponsor and the randomization code will be available from the School of Medicine Clinical Trials Office in case of emergency. If it is medically imperative to know which study treatment the subject is receiving, emergency unblinding will be done. The Director of the Clinical Trials Office will unblind the code when medically needed, without identifying other subjects' treatment. Every attempt must be made

to contact the sponsor before the code is unblinded. The person who unblinds the randomization code must record the date, time and the reason for emergency unblinding in the subject's medical record, and on the Note-to-File, and in the data management system. In such cases, the use of the investigational product must be stopped and the sponsor must be contacted immediately to determine whether the subject should be withdrawn from the study.

8.4 STATISTICAL ANALYSIS PLAN

In this chapter the most important analyses are described. Before locking the database and opening the treatment code a statistical analysis plan (SAP) document will be created, where all the analyses including the exploratory analyses will be described in detail. At least the descriptive statistics will be reported for all variables.

8.4.1 PRIMARY ANALYSIS

As the primary analysis, the incidence of rhinovirus-associated illness episodes (Yes/No) between the treatment groups will be investigated. The null hypothesis to be rejected is that there is no relationship between the treatment and the incidence of rhinovirus-associated illness episodes. The hypothesis will be tested by using a chi-square test. In case the cell frequencies, such as the expected number of subjects experiencing an illness episode or the expected number of subjects without any episodes in either of the treatment groups, turn out 5 or lower, a Fisher's exact test will be used for the comparison instead, which is seen as a more suitable method with low expected frequencies.

As a sensitivity analysis a logistic regression will be computed for the same dichotomous response (incidence of rhinovirus-associated illness episodes) but in addition to the treatment effect also adjusted with potential confounding factors. Potential confounding factors can be such as antibody status, amount of virus given on day 0, gender or incidence of wild-type virus.

In the primary analysis a p-value ≤ 0.05 is considered as significant.

8.4.2 SECONDARY ANALYSIS

The secondary analyses will be concentrating on duration of illness, incidence of infection, viral load during infection and proportion shedding the virus.

The duration of illness between the treatment groups will be investigated by comparing the group mean durations with a two sample independent t-test. In case the durations are not normally distributed a non-parametric Wilcoxon-Mann-Whitney test will be used for the comparison.

The dichotomous variables, proportion of infection incidence and virus shedding (days 1-5), will be both evaluated exactly the same way as the primary response (described in 8.4.1).

The quantitative viral titer load during infection will be evaluated with repeated measures analysis of variance model (RM-ANOVA) including the daily and overall (study days 1-5) titer load as a response, treatment, day and the interaction of day and treatment as explanatory

variables. The random effect of subject will be taken into account in the model. Model assumptions will be checked with model diagnostics and normality plots.

The secondary analyses are ordered in a hierarchical order by their clinical importance as described in 6.3. Thus no multiplicity correction is needed and a p-value ≤ 0.05 can be stated as statistically significant as long taking into account the hierarchy with the conclusions.

8.5 STUDY POPULATIONS

The study population for this study will be healthy adult (18-60 y) volunteers. The volunteers will be recruited from the University of Virginia community.

8.6 DATA MANAGEMENT

The handling of data will comply with applicable regulatory guidelines. A more detailed data management plan (DMP) for the activities described below will be prepared.

Data will be collected from the study visits using Viedoc™ Clinic (PCG Solutions AB, Uppsala, Sweden) electronic case report form (eCRF) system. Edit checks will be defined and programmed in the eCRF system to reveal possible discrepancies (missing, incomplete or illogical data) according to an agreed data validation plan (DVP). eCRF system will be used for recording data from each subject meeting the eligibility criteria and being included in the study, and for subjects who fail to meet eligibility criteria (i.e., screen failures) up to non-eligibility decision. The study staff responsible for entering data into the eCRF system will be trained by the Study Monitor prior to the site initiates subject enrollment. A personal log-in will be provided for all responsible personnel to allow for an audit trail relating to the study data. The list of responsible persons in this study alongside their responsibilities will be documented and stored in the Trial Master File (TMF). No real-time study data will be transferred via the eCRF system until the site has been qualified through the training and completion of a test eCRF before “go-live”.

Evaluations performed shall be entered in a timely manner into the eCRF by the site staff with delegated responsibility for this specific task. It is the responsibility of the site staff and Study Monitor to ensure that the eCRFs are properly completed. The data in the eCRFs should be consistent with the relevant source documents. The Investigator will sign the eCRF data entry screens to confirm that the captured data on each screen is accurate and complete. All data in the eCRF system must be stored in an unidentifiable form and treated with strict confidentiality in accordance with applicable data protection regulations.

Captured data will be monitored and reviewed electronically. Any inconsistencies will be presented as queries; either automatically generated queries raised by the automated edit checks of the eCRF system, or by manually generated queries raised by the Study Monitor and Data Manager. Queries shall be resolved in a timely manner by the trained site staff. The eCRF may contain source data for certain assessments as agreed in writing between the Study Monitor and the staff at the study site.

Recorded adverse event, medical history and concomitant medication data will be coded according to MedDRA and WHO ATC/DDD Index.

The clean database will be locked after all data have been entered, queries have been resolved/closed and the database updated accordingly.

9 REGULATORY AND ADMINISTRATIVE PROCEDURES

9.1 INSTITUTIONAL REVIEW

Prior to study initiation the protocol and ICF will be reviewed and approved by the Institutional Review Board for Health Sciences Research (IRB-HSR) at the University of Virginia.

9.2 SUBJECT INFORMATION/ INFORMED CONSENT

Subjects will be informed of the nature of the study, its aim, its possible risks and restrictions, its duration. The protocol will be explained during a meeting prior to the study and each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time. The investigator or his staff must explain to potential subjects or their legal representatives the aims, methods, reasonably anticipated benefits and potential hazards of the trial and any discomfort it may entail. They will be told that refusal to participate in the study will not prejudice future treatment. They will also be told that their study records may be examined by competent authorities and authorized persons but that personal information will be treated as strictly confidential and will not be publicly available. Subjects must be given the opportunity to ask questions. After this explanation and before entry into the trial, consent should be appropriately recorded by means of the subject's or his/her legal representative's dated signature. If a subject and his/her legal representative are unable to read, an impartial witness must be present during the entire informed consent discussion. The signature of the impartial witness will certify the subject's consent. The subject should receive a signed and dated copy of the informed consent form. No subject can enter the study before his informed consent has been obtained.

The information sheets and original signed informed consent form will be retained by the investigator. It will be kept for 15 years by the investigator and a copy will be given to the subject. The completed “subject screening log” will be signed by the investigator to attest that consent has been obtained from all subjects. Before being enrolled in the clinical study, subjects must consent to participate after the nature, scope, and possible consequences of the clinical study have been explained in a form understandable to them.

An informed consent document that includes information about the study will be prepared and given to the subject. This document will contain all the elements required by the ICH E6 Guideline for Good Clinical Practice and any additional elements required by local regulations. The document must be in a language understandable to the subject and must specify who informed the subject. Where required by local law, the person who informs the subject must be a physician.

After reading the informed consent document, the subject must give consent in writing. The subject's consent must be confirmed at the time of consent by the personally dated signature of the subject and by the personally dated signature of the person conducting the informed consent discussions. The copy of the signed consent document must be given to the subject. The original signed consent document will be retained by the investigator. The investigator will not undertake any measures specifically required only for the clinical study until valid consent has been obtained.

The investigator should inform the subject's primary physician about the subject's participation in the study if the subject has a primary physician and if the subject agrees to the primary physician being informed.

9.3 SUBJECT CONFIDENTIALITY

Subject names will not be supplied to the sponsor. Only the subject number will be recorded in the eCRF, and if the subject name appears on any other document (e.g., laboratory report), it must be obliterated on the copy of the document to be supplied to the sponsor. Study findings stored on a computer will be stored in accordance with local data protection laws. The subjects will be informed that representatives of the sponsor, independent ethics committee (IEC)/ institutional review board (IRB), may inspect their medical records to verify the information collected, and that all personal information made available for inspection will be handled in strictest confidence and in accordance with local data protection laws. The investigator will maintain a personal subject identification list (subject numbers with the corresponding subject names) to enable records to be identified.

9.4 GCP AND RECORD RETENTION

9.4.1 The study will be carried out in accordance with:

- The text of the Declaration of Helsinki adopted by the World Medical Assembly in June 1964, amended in Tokyo, October 1975, in Venice, October 1983, in Hong-Kong, September 1989, in Somerset West, October 1996 and in Edinburgh, October 2000, updated with the clarification note, Washington 2002, Tokyo 2004 and Seoul 2008.

- The ICH recommendations: Good Clinical Practice (E6), applied since January 17th, 1997.

9.4.2 Record Retention

In compliance with the ICH/GCP guidelines the investigator/institution will maintain all eCRF data and all source documents that support the data collected from each subject, and all trial documents as specified in Essential Documents for the Conduct of a Clinical Trial and as specified by the applicable regulatory requirement(s). The investigator/institution will take measures to prevent accidental or premature destruction of these documents. Essential documents must be retained until at least two years after the last approval of a marketing application in an ICH region or at least two years have elapsed since the formal discontinuation of clinical development of the investigational product. These documents will be retained for a longer period if required by the applicable regulatory requirements or by an agreement with the sponsor. It is the responsibility of the sponsor to inform the investigator/institution as to when these documents no longer need to be retained. If the responsible investigator retires, relocates, or for other reasons withdraws from the responsibility of keeping the study records, custody must be transferred to a person who will accept the responsibility. The sponsor must be notified in writing of the name and address of the new custodian.

9.5 MONITORING AND QUALITY CONTROL

9.5.1 Monitoring

The investigator will allow the representative of the sponsor and the study monitor:

- To inspect the site, the facilities and the material used for the study,
- To meet all members of the team involved in the study,
- To consult all the documents relevant to the study,
- To check that the eCRFs have been correctly completed,
- To have direct access to source documents for comparison of data therein with the data in the eCRFs,
- To check that AE have been documented,
- To verify that the study is carried out in compliance with the protocol.

This study will be monitored at regular intervals, by mutual agreement of the investigator and Monitor. All information dealt with during these visits will be treated as strictly confidential.

The investigator will provide the sponsor with the following:

- Progress reports at regular intervals,
- Adequately completed eCRFs.

Monitoring will be done according to the monitoring manual by a representative of the sponsor (study monitor) who will check the eCRFs for completeness and clarity, and cross-check them with source documents. In addition to the monitoring visits, frequent communications (letter, telephone, fax, e-mail), by the study monitor will ensure that the investigation is conducted according to protocol design and regulatory requirements. Study close-out will be performed by the study monitor upon closure of the study.

9.5.2 Audit-Inspection

The investigator will be informed that an audit may be carried out, at the request of the sponsor, before, during or after the study. The investigator will be informed that the Regulatory Authorities may also carry out an inspection. In this case, the investigator must inform the sponsor as soon as he receives the notification of inspection.

The investigator must allow the representatives of the Regulatory Authorities and persons responsible for the audit to:

- Inspect the site, facilities and material used for the study,
- Meet all members of his team involved in the study,
- Have direct access to study data and source documents,
- Consult all the documents relevant to the study.

Domestic and foreign regulatory authorities, the IEC/IRB, and an auditor authorized by the sponsor may request access to all source documents, eCRF data, and other study documentation for on-site audit or inspection. Direct access to these documents must be

guaranteed by the investigator, who must provide support at all times for these activities. Medical records and other study documents may be copied during audit or inspection provided that subject names are obliterated on the copies to ensure confidentiality.

9.5.3 Quality Control

The investigator or the appointed persons agree to complete the subject's eCRFs, at each investigation. Only the investigator or appointed persons in his team may fill out or correct the eCRFs. The eCRFs will display the subject number corresponding to the order of inclusion in the study. All corrections and alterations of data on the eCRFs must be made by the investigator or by the appointed persons. It is the responsibility of the monitor to make certain that all data are completed on the eCRFs.

At the end of each study period, the investigator must sign the eCRF in order to attest respectively to:

- Authenticity of the data collected in the eCRF ,
- Coherence between the data in the eCRF and those in the source documents.

At the end of the study, the investigator will keep a copy of the correctly completed eCRF data for his own records. Archiving of the original eCRF data will be a responsibility of the sponsor.

The investigator will keep a log of study volunteers screened for study participation and as appropriate, will indicate the reason individual study volunteers did not enter the study.

No change made in the medical files by the investigator should obscure the original information. The record must clearly indicate that a change was made and clearly provide a means to locate and read the prior information. The investigator will save data at regular intervals.

The investigator must guarantee the safety of the study data in the medical files by implementing security measures to prevent unauthorised access to the data and to the computer system.

9.6 INSURANCE AND LIABILITY

Terms will be included in the research agreement.

9.7 STUDY REPORT

9.7.1 Clinical Study Report

The results of the study will be reported in a Clinical Study Report (CSR). This report will be prepared in co-operation with DuPont Nutrition and Health, Kantvik Active Nutrition team. In compliance with the regulations, the final report will be produced within one year of completing the study. The final report will be provided to all investigators having included subjects in the study. The CSR will be provided in printing and as Word and PDF files.

9.8 PUBLICATION AND DATA RIGHTS

Terms for scientific publications are recorded in Research Agreement.

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11 Signatures

Principal Investigator

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Ronald B Turner

Date: August 17, 2016

Sponsor Representative

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Markus Lehtinen

Date