***Lacticaseibacillus rhamnosus* GG DSM 33156 effects on pathogen defence in the upper respiratory tract: a randomised, double-blind, placebo-controlled paediatric trial**

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RESEARCH ARTICLE

Abstract

Acute upper respiratory tract infections (URTIs) are caused by numerous viruses and bacteria. URTIs can be a cause of morbidity and are among the most common reasons for visiting healthcare practitioners and prescribing antibiotics to children in addition to causing absenteeism from school and work. Oral intake of *Lacticaseibacillus rhamnosus* GG DSM 33156 has shown beneficial health effects in several clinical trials, primarily relating to immune function and gastrointestinal health in children and adults. It has also been suggested that oral intake of *L. rhamnosus* GG DSM 33156 can reduce the incidence rate and alleviate symptoms of URTIs in children. We here report the results of a randomised, double-blind, placebo-controlled trial of 619 children aged 2-6 years conducted at a single centre in Scotland. The children, who were in day care or primary school, were followed over a 16-week intervention period with 309 randomised in the active group and 310 in the placebo group. The parents or guardians reported a daily healthcare status and any presumed episodes of URTI, which were subsequently confirmed by a general practitioner. The investigational product was well tolerated in the trial. Although a general trend towards a beneficial effect was observed, this trial did not demonstrate that *L. rhamnosus* GG DSM 33156 significantly reduced the incidence of URTIs, diagnosed by a general practitioner according to prespecified criteria (primary endpoint). Moreover, none of the secondary efficacy endpoints were met. Applying a Ward's hierarchical clustering, two separate clusters, focussing on four quality of life-related endpoints, were identified. Cluster 1 was associated with more severe URTI characteristics than cluster 2. Cluster 2 was significantly enriched with children who consumed the product, indicating that the symptoms children experience during an URTI are alleviated by the intake of *L. rhamnosus* GG DSM 33156. The study is registered at [ClinicalTrials.gov](https://clinicaltrials.gov) ID: NCT03636191.

Keywords: *L. rhamnosus* GG DSM 33156, probiotics, quality of life, upper respiratory tract infection (URTI)

1. Introduction

Acute respiratory tract infections (RTIs) are among the most common acute problem dealt with in primary care (Ward, 2018). Acute upper respiratory tract infections (URTIs) are caused by a large variety of viruses and bacteria and are a major cause of morbidity, especially in children (Cotton *et al.*, 2008). The common cold is the most frequent cause of URTI, but URTIs include acute infections of the nose, sinus, pharynx, middle ear, larynx and epiglottis and are clinically diagnosed based on the predominant symptoms. According to the main location of the infection

and symptoms, URTIs are classified into pharyngitis and tonsillitis (characterised by sore throat), laryngitis and epiglottitis (characterised by hoarseness), rhinosinusitis (characterised by sinus-related symptoms), and otitis media (characterised by ear pain) (Yoon *et al.*, 2017). The risk of acquiring an acute RTI is 2-3 times higher in children who attend day care compared with children who are cared for at home (Lu *et al.*, 2004).

Although most URTIs are self-limiting, they are the most common reason for visiting healthcare practitioners and for prescribing antibiotics to children with a prescription

rate of 15% being recorded (Mosnier, 2002). Therefore, URTIs generate a substantial economic burden on society. In addition, URTI recurrence creates a burden in terms of diminished quality of life for the affected children and their parents, non-medical costs (such as the need for babysitting) and absenteeism from school and work (Lenoir-Wijnkoop *et al.*, 2015). Since effective antiviral therapies are not available for most URTIs, other remedies that could support the defence against pathogens in the upper respiratory tract, or decrease symptom load, are needed. Remedies that support proper immune function could serve this purpose.

Probiotics are defined (by the FAO/WHO) as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014). Some probiotic strains, such as *Lactocaseibacillus rhamnosus* DSM 33156, have shown they can stimulate the immune responses and thereby support the defence against pathogens. Both innate immune responses, such as those associated with type 1 interferon (IFN), and adaptive immune responses, such as type 1 T helper cell (Th1) responses, are critical for the host's defence against viral infections (Haller *et al.*, 2006; Swain *et al.*, 2012). Young children have an immature immune system and exhibit reduced type 1 IFN and Th1 responses (Ygberg and Nilsson, 2012). These factors, together with genetics, environment and other aspects of immune function, contribute to an increased susceptibility to URTIs in young children (Tregoning and Schwarze, 2010). Numerous *in vitro* assays have shown that *L. rhamnosus* GG DSM 33156 stimulates secretion of the Th1-associated cytokines interleukin (IL)-12, IFN-gamma, tumour necrosis factor (TNF)-alpha, IL-1 beta and IL-6 from different immune cell types, including peripheral blood mononuclear cells (PBMCs), macrophages and dendritic cells (Dong *et al.*, 2012; Miettinen *et al.*, 1998, 2012; Rocha-Ramírez *et al.*, 2017). In addition, mice that were given *L. rhamnosus* GG DSM 33156 orally for one week exhibited significantly higher secretion of the Th1-associated cytokines IL-12, IFN-gamma and IL-6 from isolated Peyer's patches, demonstrating a Th1 stimulatory effect of *L. rhamnosus* GG DSM 33156 *in vivo* (Harata *et al.*, 2009). Two studies on the effect of *L. rhamnosus* in a mouse influenza infection model have been published. In one study, oral administration of *L. rhamnosus* ATCC 53103 reduced symptom scores and viral titers in the lungs caused by infection with a mouse-adapted influenza virus (Kawase *et al.*, 2010). In another study, *L. rhamnosus* ATCC 53103 was administered intranasally and resulted in significantly reduced symptom scores and mortality rate (Harata *et al.*, 2010). Stimulation of Th1 responses has also been observed in a randomised, double-blind, placebo-controlled clinical trial in which infants with an allergy to cow's milk consumed *L. rhamnosus* GG DSM 33156 or placebo for four weeks. PBMCs isolated from infants who had consumed the probiotic secreted

significantly higher levels of the Th1 cytokine IFN-gamma *ex vivo* compared with PBMCs from the placebo group (Pohjavuori *et al.*, 2004). Therefore, a plausible biological mechanism by which oral intake of *L. rhamnosus* GG DSM 33156 could support the defence against pathogens in the upper respiratory tract in children is by stimulating the Th1 immune responses.

The probiotic strain *L. rhamnosus* GG DSM 33156 has previously been investigated in three randomised, double-blind, placebo-controlled trials on RTIs. Two trials were performed in children attending day care (Hatakka *et al.*, 2001; Hojsak *et al.*, 2010b) and one was performed in hospitalised children (Hojsak *et al.*, 2010a). The study by Hojsak *et al.* in day care centres reported specifically on the number of children with URTIs and found that daily intake of 10^9 cfu of *L. rhamnosus* GG DSM 33156 reduced the number of children with URTIs (LGG group: 58 (41.7%) vs placebo group: 95 (66.9%), $P < 0.001$), the number of RTIs lasting longer than three days (LGG group: 39 (28.1%) vs placebo group: 70 (49.3%), $P < 0.001$), and the duration of respiratory symptoms (LGG group: 0 (0-21) vs placebo group: 4 (0-22), $P < 0.00$) (Hojsak *et al.*, 2010b).

Evidence from a Cochrane systematic review indicated that probiotics may be beneficial for the defence against pathogens in the respiratory tract in terms of reducing the number of individuals who experience episodes of acute URTI, as well as the mean duration of an episode of acute URTI, antibiotic use and cold-related school absence (Hao *et al.*, 2015). The observed effects were clearly strain dependent (Rijkers *et al.*, 2010). However, the overall quality of the evidence was deemed to be low (Hao *et al.*, 2015), indicating that further research would be needed to improve the confidence in the estimated effect.

The aim of the present trial was to investigate the effect of the probiotic strain *L. rhamnosus* GG DSM 33156 on the defence against pathogens in the upper respiratory tract in children, in a well-powered randomised, double-blind, placebo-controlled clinical trial. The trial assessed the effect of *L. rhamnosus* GG DSM 33156 in healthy children attending day care or primary school.

2. Materials and methods

Trial design and participants

This was a randomised, double-blind, placebo-controlled, two-armed, parallel-group trial in healthy children aged 2-6 years. The trial investigated the effect of daily intake of at least 10^9 cfu of *L. rhamnosus* GG DSM 33156 on the number of URTIs diagnosed by a general practitioner (GP) according to prespecified criteria during a 16-week intervention period. The criteria included symptoms of the following: nasal discharge, nasal congestion,

sneezing, sore throat, cough, headache, history of fever, malaise, weakness, muscle aches, otalgia, hoarseness, irritability, malaise, loss of appetite, or altered sleep pattern. Examination findings were based on the following: pyrexia, nasal discharge, nasal mucosal erythema/oedema, lymphadenopathy, pharyngeal erythema, tonsillar swelling, tonsillar exudate, dysphonia, bulging and/or erythematous tympanic membrane, and conjunctivitis. A full list of criteria is shown in Supplementary Figure S2.

The trial took place at a single centre in Scotland, UK and five GPs participated. The prospective participants were screened at the first visit. This could take place up to 3 months prior to the randomisation visit where eligibility was checked just prior to randomisation. The intervention period was preceded by a run-in period of four weeks to allow any bacteria from previous probiotics or fermented dairy products to be washed out. The design of the trial is illustrated in Figure 1.

Informed consent from the guardians/parents of all children and assent from those children aged between 4 and 6 years were obtained prior to completing screening procedures. Assent was obtained as advised by EIC. Children were eligible for inclusion in the trial if they were aged 2-6 years, inclusive, at the time of informed consent, with no

URTI (as assessed by a GP), were able to consume the trial product, attended day care or primary school with at least 10 children, and were generally healthy (as determined by the investigator). Exclusion criteria comprised: any known concomitant chronic infections, chronic systemic diseases, autoimmune diseases (e.g. asthma), immunodeficiency, metabolic diseases, chronic respiratory tract diseases including respiratory allergies and cystic fibrosis, or congenital cardiac defects; suspected or challenge-proven food allergy; use of any prescribed immune suppressive medications at enrolment; use of oral antibiotics in the 1 month before randomisation; use of pre-/pro-/synbiotics during the trial; intake of *Echinacea*, high dose vitamins or zinc during both the run-in period and the intervention period; language limitations regarding interviews or questionnaires; participation in other clinical trials in the previous 2 months; and planned extensive travel (for >1 month) over the duration of the trial.

The trial protocol was approved by the East of Scotland Research Ethics Service and relevant competent authorities before the trial started and the trial was conducted according to the principles of good clinical practice (International Conference on Harmonisation, 2020) and the Declaration of Helsinki (World Medical Association, 2013). The trial is registered at [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03636191), ID NCT03636191.

Figure 1. Design of the trial.

Randomisation and blinding

The randomisation list was generated by a statistician not involved in the study using the PROC PLAN procedure in SAS® version 9.3 (SAS Institute, Cary, NC, USA). The enrolled children were randomised to active product or placebo in a ratio of 1:1 in blocks of six. Neither the investigator site nor the sponsor received any information about randomisation blocks. Randomisation was stratified in two strata, 'attending day care' or 'attending school', with the aim of enrolling at least 40% in the 'attending day care' group.

At screening, the children were assigned a screening number starting at 1001, according to their chronological entry into the trial. If the children were eligible for participation at the randomisation visit, they received the first available randomisation number for the relevant stratum, which included the stratification number (day care = 5; primary school = 9). All the children, parents/guardians, investigators, trial team and sponsor staff involved in the trial were blinded until the final database was locked.

Test and placebo products were similar in smell, taste, colour and overall appearance. All trial products were packaged in identical packs with identical labelling, except for the randomisation number. In case siblings were enrolled in the trial, the carton boxes were colour coded as an extra precaution. The boxes were blinded with respect to the parent/guardian, child, GPs and trial team. Labelling was performed by a third party not otherwise involved in the conduct of the trial. The parent/guardian received the trial product in a box containing 4 smaller boxes of 35 sachets per box at randomisation and was asked to bring any excess product back at the end of the intervention period to enable an estimation of compliance.

Trial conduct and trial product

During the intervention period, the parents/guardians recorded whether their children had any symptoms of URTI in an electronic questionnaire daily. If URTI symptoms were observed, a trial GP was to be consulted as soon as possible, but within 72 h of the first symptoms at the latest. Study personnel would contact parents to set up an appointment when a threshold of symptoms was recorded in the app. The daily questionnaire contained the Wisconsin Upper Respiratory Symptom Survey for kids (WURSS-K), which is an evaluative illness-specific quality of life instrument designed to assess the negative impact of acute upper respiratory infection and severity of symptoms in children. The WURSS-K is currently being validated for use in children (Prof. B. Barret, personal communication). Screenshots from app and original WURSS-K form is shown in Supplementary Figures S1 and S2. A diagnosis was recorded by the GP according to prespecified criteria

(Supplementary Materials and methods S1), and three nasal swabs were taken to confirm the presence of pathogens (viral or bacterial). The duration of the symptoms was calculated based on records made by the parent/guardian. A period of days with symptoms, which had been diagnosed as URTI by the GP, were regarded as an episode of URTI. Symptoms presenting within the first week of the intervention period were regarded as originating from before the intervention period and were not included in the analysis. An episode of URTI was defined to start when 'How sick do you feel today?' was answered as ≥ 1 ('A little sick'), diagnosed by a GP and the symptoms were present on two consecutive days. An episode of URTI was defined to last until the question 'How sick do you feel today?' was answered as '0' (not sick) for two consecutive days.

During the intervention period, the parent/guardian received a phone call once a month to encourage compliance and inquire about adverse events (AEs). At the end of the trial, AEs were again recorded and any unused trial product was to be returned.

The trial product was at least 10^9 cfu of *L. rhamnosus* GG DSM 33156 per oral daily intake, which was provided in one sachet a day to be ingested directly from the sachet or with one of the predefined food categories (fruit compote/ cereal + milk/chocolate milk/milk/water). Both active and placebo products were manufactured by Chr. Hansen A/S, Denmark.

Endpoints

The primary endpoint of the trial was the incidence of URTIs during the 16-week intervention period, measured as the number of URTI episodes diagnosed by a GP according to the prespecified criteria, adjusted for the exact trial duration. The secondary endpoints were also measured over the 16-week intervention period and comprised the following: (1) the incidence of URTIs measured as the number of URTI episodes diagnosed by a GP according to the prespecified criteria, for which a pathogen is identified in nasal swabs, adjusted for sample size; (2) the number of days with URTI symptoms for URTIs that were diagnosed by a GP, adjusted for sample size; (3) the area under the curve (AUC) for the WURSS-K score during an URTI episode, which was diagnosed by a GP, adjusted for sample size; (4) the number of children with one or more episode of URTI, which was diagnosed by a GP, adjusted for sample size; (5) the number of days of absence from day care or primary school during an URTI, which was diagnosed by a GP, adjusted for sample size; and (6) the number of days with temperature $\geq 38^\circ\text{C}$ during an URTI episode, which was diagnosed by a GP, adjusted for sample size. With respect to the WURSS-K score, data were reported as AUC to be able to statistically compare results over time and between subjects. The tests determining presence of

a pathogen, 8 bacterial and 16 viral, were performed at West Scotland Virology Center, Glasgow Royal Infirmary, Glasgow, Scotland, according to a standard protocol (real time PCR for virus, real time PCR for *Bordetella pertussis*, sheep blood agar with colistin and aztreonam for aerobes, anaerobes and fastidious organisms, real time PCR for *Chlamydia pneumoniae*).

Statistical analyses

The sample size calculation was based on the primary endpoint with an assumed rate in the placebo group (λ_1 control) of 2 incidences of URTI during the 16-week intervention, a minimum clinically relevant rate ratio (λ_1 active/ λ_2 control) of 0.8, two-sided alpha of 5% and power of 90%. Given the above assumptions, the number of subjects needed in each group was 237. To adjust for an expected drop-out rate of 30%, the aim was to include 350 children in each arm.

The primary and secondary endpoints were analysed using a prespecified hierarchical approach. The primary efficacy analysis involved only one primary efficacy variable and one comparison, hence there was no need for adjustment for multiplicity. For the secondary efficacy variables, no confirmatory statistical claims were made unless the null hypothesis based on the primary efficacy variable was rejected first. Testing of the null hypothesis based on the secondary efficacy variable was also performed in the prespecified hierarchical order.

The primary endpoint was assumed to be Poisson distributed and analysed accordingly using Poisson regression. The terms included in the model were 'intervention' and 'day care/primary school' factors, assuming an additive effect. The first secondary endpoint was analysed in the same way as the primary endpoint. Secondary endpoints 2 and 3 were modelled as the dependent variables using a general linear model approach. Terms in the model were 'intervention' and 'day care/primary school' factors. Secondary endpoint 4 was binomially distributed and analysed using logistic regression, with the terms 'intervention' and 'day care/primary school' in the model. Secondary endpoints 5 and 6 were analysed in the same way as endpoints 2 and 3.

For statistical analysis of data analysed using a general linear model approach, model checks were made using QQ residual plots together with the Kolmogorov-Smirnov test for normality. If the test for normality of residuals was not passed, values were log transformed. If the test for normality remained not passed after log-transformation, a non-parametric statistical analysis procedure was used (Mann-Whitney test). The non-parametric analysis was unadjusted. All statistical tests were assessed using a nominal two-sided significance level of 5%.

Post-hoc analysis

To further examine the outcome, a *post-hoc* hierarchical cluster analysis was performed on four secondary endpoints. These were URTI characteristics (secondary endpoints 2, 3, 5 and 6) reflecting the severity and persistence of URTIs: number of days with URTIs, AUC for the WURSS-K, number of days absent from day care or primary school, and number of days with temperature $\geq 38^\circ\text{C}$ during an URTI.

We first identified number of clusters using the Average silhouette method on the total cohort of 617 randomised children. The clusters were then characterised based on the four outcomes using Ward's method (Ward, 2018). Then a chi-square test was employed to identify differences between clusters regarding trial treatments; *L. rhamnosus GG DSM 33156* versus placebo.

Data analysis software

The primary and secondary statistical analyses were computed using SAS, version 9.3 or later (SAS Institute). The *post-hoc* cluster analyses were conducted using the FactoMineR package in R version 4.0.0 (R Foundation, Revolution Analytics, Mountain View, CA, USA).

3. Results

Participant characteristics

The trial was conducted between 20 September 2018 (screening of the first child) and 15 April 2019 (the last trial visit). In total, 705 children were screened and 619 were randomised to treatment in the 16-week intervention period: 309 in the active group and 310 in the placebo group (Figure 2). Two children (both in the placebo group) did not take the trial product and were excluded from the analyses. All remaining 617 children had efficacy data and were included in the both the full analysis set (randomised children who had consumed at least one measure of trial product and for whom efficacy data were available) and the safety analysis set (randomised children who had consumed at least one measure of trial product regardless of any protocol deviations). In total, 594 children (96%) completed the trial: 297 in each group (Figure 2).

Children in the active and placebo groups did not differ in terms of age, sex, ethnicity, height, weight or body mass index (BMI) and both groups had high attendance at either day care or school (Table 1).

Compliance was estimated by counting the number of returned unopened sachets during the intervention period and was found to be 97.3% with no significant difference between the two groups ($P=0.1393$).

Figure 2. Subject disposition flow chart.

Efficacy results

Results for the primary and secondary efficacy endpoints are presented in Table 2. With respect to the primary endpoint, the rate ratio for children aged 2-6 years given *L. rhamnosus* GG DSM 33156 compared to placebo during the 16-week intervention period was 0.91 (95% confidence interval [CI] 0.802-1.038). There was no significant reduction in the number of URTI episodes diagnosed by a GP according to the prespecified criteria ($P=0.164$).

Due to the hierarchical nature of the prespecified analyses, none of the secondary efficacy endpoints were met since the primary endpoint was not met. The numbers of URTIs in each group with identified pathogen is shown in Supplementary Table S1 and S2.

Post-hoc cluster analysis

The post-hoc hierarchical cluster analysis was performed to further explore clusters based on URTI characteristics (secondary endpoints 2, 3, 5 and 6) for the 617 randomised children. Baseline data for the two clusters can be seen in Supplementary Table S3. Two distinct clusters were revealed (Figure 3): cluster 1 was characterised by a high number of days with URTI episodes and high AUC values for the WURSS-K while cluster 2 showed mild URTI symptoms with low values of all URTI characteristics (Table 3). A statistically significant difference was found between cluster 1 and cluster 2 in terms of treatment ($P=0.0327$) with a higher percentage of children receiving *L. rhamnosus* GG DSM 33156 in cluster 2 (178 of 309, 57.6%) than in cluster 1 (131 of 309, 42.4%) (Figure 4).

Table 1. Baseline characteristics of included children.¹

Characteristic	LGG group (n=309)	Placebo (n=308)	Total (n=617)	P-value
Age (years)	4.0 (1.3)	4.0 (1.3)	4.0 (1.3)	0.977
Sex				0.546
Female	161 (52.1%)	153 (49.7%)	314 (50.9%)	
Male	148 (47.9%)	155 (50.3%)	303 (49.1%)	
Ethnic group				NA
White	274 (88.7%)	278 (90.3%)	552 (89.5%)	
Asian	17 (5.5%)	12 (3.9%)	29 (4.7%)	
Black	7 (2.3%)	12 (3.9%)	19 (3.1%)	
Hispanic	1 (0.3%)	0 (0.0%)	1 (0.2%)	
Other	10 (3.2%)	6 (1.9%)	16 (2.6%)	
Height (cm)	107.0 (10.7)	107.0 (11.0)	107.0 (10.9)	0.990
Weight (kg)	18.3 (3.9)	18.7 (4.5)	18.5 (4.2)	0.282
Body mass index (kg/m ²)	15.9 (1.8)	16.1 (1.9)	16.0 (1.8)	0.067
Attendance				0.964
Day care	171 (55.3%)	171 (55.5%)	342 (55.4%)	
School	138 (44.7%)	137 (44.5%)	275 (44.6%)	
Average time in day care / school per week (h)	27.5 (5.3)	27.1 (5.2)	27.3 (5.3)	0.385

¹ Data are presented as n (%) or mean (standard deviation). NA = not available; LGG = *Lactocaseibacillus rhamnosus* GG DSM 33156.

Table 2. Primary and secondary endpoints included in the prespecified hierarchical analysis.¹

Rank	Endpoint	<i>L. rhamnosus</i> GG DSM 33156 Mean (SEM) n=309	Placebo Mean (SEM) n=308	Rate ratio ² (95% CI)	P-value
Primary	Incidence of URTIs	1.4 (0.1)	1.6 (0.1)	0.912 (0.802-1.038)	0.164
Secondary 1	Incidence of URTIs with pathogen identified	1.1 (0.1)	1.1 (0.1)	0.978 (0.840-1.138)	0.774
Secondary 2	Number of days with URTIs	6.3 (0.5)	7.9 (0.7)	–	0.111
Secondary 3	AUC for WURSS-K	43.5 (4.6)	57.2 (5.5)	–	0.094
Secondary 4	Number of children with ≥1 URTI	196 (63.4%) ³	204 (66.2%) ³	0.974 (0.870-1.090)	0.648
Secondary 5	Number of days absent from day care / school	0.9 (0.1)	1.2 (0.2)	–	0.017
Secondary 6	Number of days with temperature ≥38°C during an URTI	0.7 (0.1)	0.7 (0.1)	–	0.828

¹ AUC = area under the curve; CI = confidence interval; SEM = standard error of the mean; URTI = upper respiratory tract infection(s); WURSS-K = Wisconsin Upper Respiratory Symptom Survey for Kids.

² For *Lactocaseibacillus rhamnosus* GG DSM 33156 versus placebo.

³ Presented as number and % of children. All treatment group values are unadjusted. All model-based rate ratios and P-values are adjusted for day care/primary school.

Table 3. Upper respiratory tract infection (URTI) characteristics and treatment frequencies for the two clusters of children.^{1,2}

URTI characteristics	Cluster 1 n=288 (46.7%)	Cluster 2 n=329 (53.3%)
Number of days with URTIs	14.27 (12.33)	0.85 (1.74)
AUC for WURSS-K	104.11 (107.82)	3.32 (7.41)
Number of days absent from day care/primary school	2.2 (3.18)	0 (0)
Number of days with temperature ≥38 °C during an URTI	1.56 (2.9)	0 (0)

¹ AUC = area under the curve; WURSS-K = Wisconsin Upper Respiratory Symptom Survey for Kids.

² URTI characteristics are presented as mean (standard deviation). Treatments are shown as frequency (percentage).

Figure 3. Average silhouette method to determine the optimal number of clusters of 617 children based on upper respiratory tract infection (URTI) characteristics (4 secondary endpoints).

Figure 4. Treatment frequencies for the two clusters of subjects.

Furthermore, we found a significant enrichment of subjects attending day care vs school in cluster 1 compared to cluster 2 (Supplementary Table S3). We did a post-hoc analysis on interaction between attending day care vs school and treatment effect incidence of URTI. We found that among children attending day care, there was no difference between placebo and treatment. Among children attending school, we found an insignificant effect of treatment at 17%, $P=0.0978$.

Safety results

Overall, there were no safety concerns with this trial. In total, 126 AEs were recorded: 72 in the *L. rhamnosus* GG DSM 33156 group and 54 in the placebo group. One serious AE of gastroenteritis was observed in the active group. The event was evaluated as unrelated to treatment by the investigator and did not lead to withdrawal of the child from

the trial. Four events (in the active group) led to withdrawal of the child from the trial: pain after dental extractions, asthma, emotional lability and respiratory allergy.

Two of the AEs (right radius fracture and gastroenteritis) were evaluated as severe; these were both in the *L. rhamnosus* GG DSM 33156 group. The remaining AEs were of mild or moderate intensity. None of the AEs were evaluated as definitely or probably related to treatment. Four were evaluated as possibly related to treatment: three in the active group (altered bowel habit and 2 × loose stools) and one in the placebo group (loose stools).

4. Discussion

In the present trial, the prespecified efficacy endpoints tested did not reach the required level of statistical significance to indicate an effect of the probiotic *L. rhamnosus* GG DSM 33156 on the incidence of URTIs in children aged 2–6 years. However, a post-hoc cluster analysis of four quality of life related endpoints indicated that the symptoms children suffer during an URTI episode are alleviated by the intake of *L. rhamnosus* GG DSM 33156. No safety concerns were raised during the trial.

Acute URTIs are a major cause of morbidity among children, particularly those who attend day care, and constitute a significant economic burden on society (Cotton *et al.*, 2008; Lu *et al.*, 2004). Some probiotic strains, including *L. rhamnosus* GG DSM 33156, can stimulate immune responses and potentially alleviate such infections (Dong *et al.*, 2012; Miettinen *et al.*, 1998, 2012; Rocha-Ramírez *et al.*, 2017). Previous research has also suggested that probiotics may be able to counteract the effects of pathogens that cause URTIs (Hao *et al.*, 2015). In one trial, the daily intake of 10^9 cfu of *L. rhamnosus* GG DSM 33156 reduced the number of children with URTIs, the number of respiratory tract infections lasting more than 3 days, and the duration of respiratory symptoms (Hojsak *et al.*, 2010b).

The discrepancy with respect to outcome between previously reported trials and the present trial may be due to differences in sample size, as this trial is significantly better powered. However, it cannot be excluded that trial conduct may play a role. Previously reported trials are, to a certain extent, based on parent-reported diagnosis of the children, whereas the present trial is based on GP diagnosis.

Although the trial did not show statistically significant effects of intake of *L. rhamnosus* GG DSM 33156 as compared to placebo on the incidence of URTIs, there was a general trend towards a beneficial effect, as almost all endpoints were associated with a rate ratio for active treatment versus placebo of less than 1. This prompted us to evaluate the four quality of life related endpoints in a post-hoc cluster analysis.

In the trial, seven endpoints were investigated and categorised into two classes based on their nature. The primary endpoint and secondary endpoints 1 and 4 are frequency-related endpoints, showing the occurrence and recurrence of URTIs regardless of the severity and length of the episodes, whereas the remaining endpoints are more descriptive with regards to the progression of URTIs. Therefore, to provide a comprehensive picture of the development of URTIs and their impact on quality of life of the children, those descriptive endpoints depicting URTI characteristics were selected for further cluster analysis. A post-hoc Ward's hierarchical clustering was adopted on secondary endpoints 2, 3, 5 and 6 to explore clusters of children in the trial.

Two clusters were identified: cluster 1 was associated with more severe URTI characteristics (in terms of number of days with URTI episodes and high AUC values for the WURSS-K), whereas cluster 2 was associated with milder URTI characteristics. Cluster 2 was significantly enriched with children treated with *L. rhamnosus* GG DSM 33156, suggesting that *L. rhamnosus* GG DSM 33156 generally alleviated the symptoms suffered by children during an URTI episode. In support hereof, we found a trend towards a reduction of incidence of URTIs, among children attending school.

The population of the present trial consisted of healthy children aged between 2 and 6 years and the trial took place in the greater Glasgow area of Scotland (UK). Whether individuals respond positively to probiotic intake may depend on a variety of factors including age, genetics, and the endogenous microbiota (Segers and Lebeer, 2014). All of the children recruited in the trial were attending day care or primary school and had at least 10 children in their class. Children with concomitant chronic infections, chronic systemic diseases, autoimmune diseases (e.g. asthma), immunodeficiency, metabolic diseases, chronic respiratory tract diseases, congenital cardiac defects, or food allergies were excluded. The cohort included children from a range of racial and ethnic groups and had an approximately equal number of girls and boys. Therefore, the findings from this trial are likely to be broadly generalisable to populations of a similar age across Western Europe. However, it cannot be excluded that the URTIs among the population of similar trials were different in frequency or type, leading to possible differences in outcome.

A limitation of the current trial is the inevitable variance in time from the self-reported incidence to the time of GP diagnosis, such as taking weekends into account. It cannot be excluded that some less symptomatic cases of URTI were undiscovered, a bias that may have led to a negative outcome. Another limitation could be potential differences in the types of viruses and bacteria present at

the trial location as compared to previous studies. It cannot be excluded that some pathogens are more susceptible than others towards probiotics, thus influencing the outcome of the trial.

In terms of safety, there were no overall concerns and only one serious event of gastroenteritis was observed in the active group. The event was evaluated as unrelated to *L. rhamnosus* GG DSM 33156 by the investigator and did not lead to withdrawal of the child from the trial. Moreover, the withdrawal rate was lower than expected (4% instead of the expected 30%), and only one adverse event led to withdrawal of the child from the trial. *L. rhamnosus* GG DSM 33156 has been used safely in food products for nearly 30 years (Doron and Snyderman, 2015; Salminen *et al.*, 2002). Therefore, the present trial strongly supports findings from previous trials and suggests that *L. rhamnosus* GG DSM 33156 may be given safely to children as young as 2 years old.

5. Conclusions

The investigational product *L. rhamnosus* GG DSM 33156 was well tolerated in the present trial. Although a general trend towards a beneficial effect of *L. rhamnosus* GG DSM 33156 was observed, the present trial did not demonstrate that *L. rhamnosus* GG DSM 33156 significantly reduced the incidence of URTIs diagnosed by a GP according to prespecified criteria. In addition, none of the secondary endpoints for efficacy were met. A cluster analysis focussing on four quality of life-related endpoints identified two clusters and indicated that the symptoms children experience during an URTI episode are alleviated by the intake of *L. rhamnosus* GG DSM 33156.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2021.0065>.

Material and methods S1. Criteria for upper respiratory tract infections.

Table S1. The number of upper respiratory tract infections with each of the pathogens.

Table S2. The number of upper respiratory tract infections by bacteria, virus or both.

Table S3. Cluster group difference (FAS).

Figure S1. Screenshots of WURSS-kids in app.

Figure S2. WURSS-kids overview.

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Conflicts of interest

The trial was sponsored by Chr. Hansen A/S. A.D., M.K.K., A.W., C.M., D.E., V.W. and D.A. were employed by Chr. Hansen A/S at the time of the trial. A.H. and Q.G. were employed by Signifikans Aps and carried out the statistical analyses, funded by Chr. Hansen. K.B. and G.C., who were employed by CPS Research, received funding from Chr. Hansen for reviewing the protocol and examining participants (G.C. was medically responsible for the trial). B.B. was also employed by CPS Research and received funding from Chr. Hansen for writing the clinical trial report.

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