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Effects of probiotic yogurt on relative respiratory tract infections, urine, saliva biomarkers, and fecal bacterial load in Ugandan children: a randomized controlled trial

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This study evaluated the effects of locally produced probiotic yogurt on infectious diseases in Ugandan children aged 3–6 years. Over nine weeks, 196 children participated in a randomized, double-blind, placebo-controlled trial, consuming 125 ml daily of either probiotic yogurt containing *Lacticaseibacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106 or a non-fermented dairy placebo. The primary outcome, average daily incidence of upper respiratory tract symptoms, showed no significant difference between groups. However, the probiotic yogurt group experienced a significant reduction in respiratory tract infection symptoms over time ($p = 0.02$). Biomarker analysis revealed significant changes in the probiotic yogurt group, including higher urine hippurate levels ($p = 0.02$), increased lactic acid bacteria ($p = 0.04$) and total bacterial load ($p = 0.04$) in stool, and elevated SLPI ($p = 0.005$) in saliva from baseline to endline. Despite these within-group effects, the lack of significant differences between the yogurt and placebo groups highlights the need for further research with larger cohorts and longer durations to confirm the potential benefits of this probiotic yogurt for reducing infection symptoms and improving health biomarkers under these study conditions.

Keywords Probiotic yogurt, Placebo-controlled nutrition intervention, Respiratory tract infections, *Lacticaseibacillus rhamnosus*, Fermented foods, School feeding program, Uganda

Children in sub-Saharan countries, such as Uganda, are at a relatively high risk of morbidity due to frequent microbial infections, poor sanitary conditions, crowded households, tropical climate, and malnutrition. The most recent Ugandan National Demographic and Health Survey indicated that 7–12% of children below the age of five years old suffer from respiratory tract infections (RTI's)[1]. For gastrointestinal tract infections, rotavirus is still the major pathogen in children less than five years old[2]. Among skin infections, tinea capitis (caused by the fungi *Trichophyton* or *Microsporum*) is the most incidental infection, peaking in children between three and seven years old[3].

Poor and low dietary intake can lead to reduced immunity and increased susceptibility to infections. The World Health Organization (WHO) recommends a dietary intake of 0.66 g protein/kg body weight per day[4]. As in many low-income countries, the diet in Uganda predominantly consists of carbohydrate-rich staple foods[5]. Hence, many people do not meet the recommended daily protein intake[6]. To address the problem of poor dietary intake among children and associated health markers and to leverage the high production of milk in the southwestern dairy-farm region of Uganda, the Netherlands Development Organization (SNV) designed a program called The Inclusive Dairy Enterprise Project (TIDE) to promote the consumption of milk in schools as part of a larger developmental project in the dairy sector in this region[7].

Under this program, a child receives 100 ml of milk, five days per week, during the school term of 12 weeks. After four years of implementation, approximately 300,000 primary and pre-primary school children

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were enrolled in the program. Following the success of the TIDE school milk program, SNV and Yoba for Life Foundation designed a similar program that included locally produced probiotic yoghurt instead of milk. To introduce probiotic yoghurt in the market, over 170 communities and small entrepreneurs were trained on production of probiotic yoghurt using locally sourced fresh milk[8,9].

Shifting from milk to a fermented food product, such as yoghurt, provides a number of additional benefits, including increased shelf life of the product, enhanced nutritional value from the bacterial pre-digestion of lactose and milk proteins, production of vitamins, and propagation of viable bacteria with potential health benefits[9,10]. Probiotic bacteria, including *Lacticaseibacillus rhamnosus* yoba 2012, the generic version of *Lacticaseibacillus rhamnosus* GG[11], have been shown to alleviate infections that frequently occur in young children. The bacterium *L. rhamnosus* GG is the world's best documented probiotic with a number of proven health benefits[12], including the prevention and reduction of diarrhea[13], common cold[14], allergies, and skin conditions[15]. More than 30 studies have been conducted on the effect of probiotics on children attending day care centers, with mostly the incidence of gastrointestinal infections and respiratory tract infections as health outcomes[16]. No adverse effects of the consumption of *L. rhamnosus* GG have been reported in children.

Notwithstanding the large body of evidence showing the ability of *L. rhamnosus* GG to prevent and reduce the above-mentioned conditions, the efficacy of *Lacticaseibacillus rhamnosus* has not been extensively studied in the population of Ugandan children. However, since the introduction of the probiotic starter culture containing *Lacticaseibacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106, probiotic yoghurt has been produced in many regions of the country. Part of the locally produced yogurt has been introduced in school feeding programs, mainly in pre-primary schools[8,17–19]. This infrastructure has facilitated the study of the health benefits of the daily intake of this locally produced probiotic yoghurt in this population group in a real-life setting.

The present study is a follow-up of a previously described study including 1116 school children aged three to six years old in Southwest Uganda attending ten different schools consuming either probiotic yoghurt or milk[7]. The results of this non-randomized study suggested beneficial effects of the consumption of probiotic yoghurt fermented by *L. rhamnosus* yoba 2012 and *S. thermophilus* C106 on the incidence of skin diseases (tinea capitis) and respiratory tract infections. The limitations of the previous study prompted the need for a randomized double-blind, placebo-controlled follow-up study, as described in this paper.

The present study was conducted with 196 children aged three to six years old in a school in Southwest Uganda. During the study, all the children were randomized and distributed across four classes. This study included respiratory tract infection (RTI) symptoms as the primary health outcome. Immune markers in saliva, metabolites in urine, and bacterial load in stool samples were included as objective health markers and secondary outcome measures. Other secondary outcome measures were incidence of skin infection symptoms, incidence of diarrhea, anthropometric indicators, and absenteeism. Only children whose parents provided consent and agreed to pay for yoghurt or milk consumption as part of the ongoing school feeding program participated in the study.

Methods

Subjects and design

The study followed a controlled interrupted time-series design, carried out over a period of 12 weeks, and included 196 children (intention to treat population) aged three to six years old from one school in Sheema District, Southwest Uganda. Randomization into two arms was performed with the help of www.randomlists.com/team-generator. This study compared markers for common infectious diseases among children consuming 125 ml of probiotic yoghurt containing *Lacticaseibacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106 with children consuming 125 ml of a non-fermented placebo dairy product. Consumption frequency was daily for five days per week during the eight weeks of intervention, following a two to three weeks baseline period. Prior to the study, written informed consent was obtained from the parents or caregivers of all participating children. The study was reviewed and approved by the Research Ethics Committee of the Mbarara University of Science & Technology (Mbarara, Uganda), study reference 01/09–18. All methods were performed in accordance with its relevant guidelines and regulations. This trial has been registered at ClinicalTrials.gov (NCT04144491).

Intake of dairy products and blinding

The probiotic yoghurt was produced by a local rural producer (K-Yoba in the Sheema district) according to the protocol as described by Westerik *et al.*[9] The yoghurt was made by using a starter culture containing *Lacticaseibacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106. The final yoghurt product contained 5% (w/v) sugar and 0.1% (w/v) artificial flavors (both strawberry and vanilla flavors were used for variation during the study). In accordance with the cell count analysis described by Kort *et al.*[17], the final product was expected to contain approximately 1×10^{10} CFU *L. rhamnosus* yoba 2012 and 1×10^{11} CFU *S. thermophilus* C106 per 125 ml of product. As a placebo, custard was made from milk including sugar 5% (w/v), corn-starch 4% (w/v), and artificial flavors (for variation strawberry or vanilla, 0.1% (w/v)). The products were packed and served in small polyethylene bags of 125 ml, which is common practice in Uganda.

The products were delivered at the school in a blue box (group 1) and a pink box (group 2) on a daily basis around noon. Teachers, pupils, school nurses, and researchers did not know which group received which product. Before consumption of the product and to prevent sharing of the product with pupils belonging to the other group, children from 'team pink' and 'team blue' were separated and seated on opposite sides of the classroom. The products were consumed through a straw. Pupils were encouraged to finish the entire package, but in the incidental case that they failed to do so, the leftovers were discarded. Waste bins were provided to the schools for plastic bag disposal. Unblinding of groups 1 and 2 was performed after data analysis.

Parent questionnaires

Three times during the study, in week one, five and ten (Figure 1) the parents of the participating children were asked to complete a questionnaire as reported previously[7]. The objectives of this questionnaire were to collect data about 1) the socio-demographic characteristics of the children that might affect the study outcomes, 2) the diet of the child, specifically regarding the intake of dairy products and fermented foods. Dietary information was collected using the Dietary Diversity Score (DDS) tool. This standard tool for nutritional surveys has been developed to assess the variety of foods that a household accesses and consumes on an average day[20]. Food diversity is scored between 0–12, based on a listing of food consumed in the last 24 hours from a maximum of 12 different food groups.

Absenteeism

Teachers in every school kept track of child absenteeism with the help of specifically designed attendance lists, which were subsequently digitalized by project team members.

Incidence of common cold and skin conditions

Two external nurses and one school nurse visited the schools five days per week to monitor the health status of each child. At the beginning and end of the study, the nurses collected anthropometric data and urine, saliva, and stool samples (Figure 1). The nurses focused on symptoms of common cold and recorded incidences of cough, rhinitis (running nose), and skin rashes (tinea capitis). The nurses were equipped with tablets that contained the previously reported software application (app) (Kenga Mobile, OMNI-Tech Ltd, 2018) for digital data recording[7]. A picture of the affected body part was taken and linked to the app questionnaire. No therapeutic actions were taken by the nurses.

Incidence of diarrhoea

Parents were called by phone on a weekly basis and asked whether their child had suffered from diarrhea, defined as three or more loose stools per day. The incidence of diarrhea was subsequently recorded as a binominal value (yes/no) weekly.

Anthropometric indicators

Anthropometric data were collected from the children at the beginning and end of the term and their weights were measured using a digital weighing scale (Casa CEGS01, South Africa). The height of the children was measured using a floor-standing height rod (Fazzini S225; Italy). All equipment was calibrated, and the nurses were collectively trained, individually coached, and monitored during their activities. The training and coaching of the nurses were performed according to the WHO Training Course on Child Growth Assessment[21] by the study coordinator.

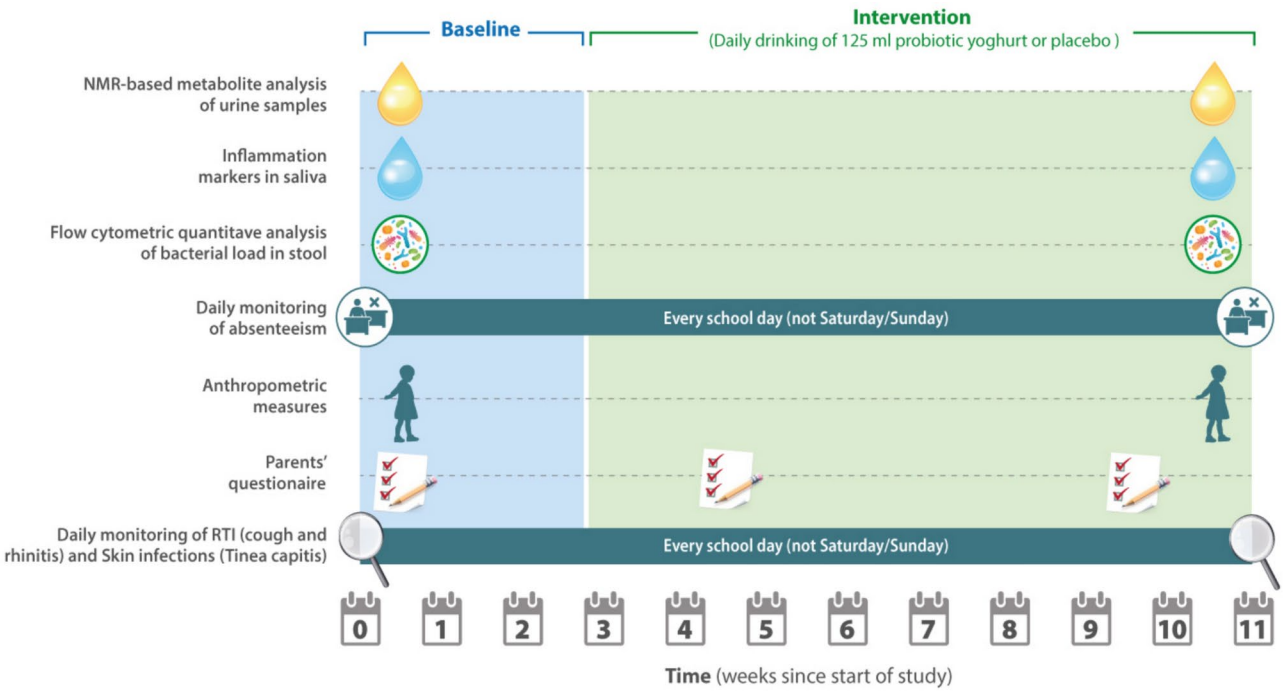

Fig. 1. Experimental design of the randomized double-blind placebo-controlled intervention study with probiotic yoghurt.

Collection of urine samples

Morning urine from each child was collected in an 80-ml container once during the baseline period and once during the last week of the study. The volume of urine and time of sample collection were recorded. Within 1.5 hours from the time of collection, samples were pipetted in triplicate into 1.5 ml cryovials and stored at −20°C for a maximum of two weeks. Subsequently, the samples were transferred to a −80°C deep freezer and stored until analysis.

Collection of stool samples

Stool samples from each child were collected once during the baseline period and once during the last week of the study period. To avoid urinating the stool sample, the child was asked to urinate before collection of the stool sample. Children defecated while squatting on a sheet of paper on the floor of the toilet block. After donation, each stool sample was transferred in quadruplicate to 1.5 ml cryovials with the help of small wooden applicator sticks. The samples required for future cell cultivation were collected in a cryovial containing 15% glycerol and 85% normal saline solution (0.9%). Within one hour from the time of collection, samples were stored at −20°C for a maximum of two weeks. Subsequently, each sample was transferred to a −80°C deep freezer and stored for further microbiological analysis.

Collection of saliva samples

Saliva samples from each child were collected once during the baseline period and once during the last week of the study period. Samples were collected after the children had fasted for one hour. Ten minutes before sample collection, each child was given water to drink. Specially designed sticks with a sponge at one end (Oracol, Malvern Medical Developments Limited, United Kingdom) were used for sampling. The sponge was placed between the cheek and lower teeth of the child for five minutes. Subsequently, the sponge was removed from the child's mouth, placed in its corresponding cover tube, and immediately stored at −20°C for a maximum of two weeks. Subsequently, the samples were transferred to a −80°C deep freezer and stored until analysis.

Data recording

Data were collected and recorded during school days. Data were not collected during weekends, public holidays, or days when the school was closed due to special circumstances (e.g., exam period for higher classes). Following the start of the school term, the majority of the children had not yet attended school during the first week. Hence, the baseline data were only calculated in the second and third weeks of the study until the start of the intervention in the fourth week. Similarly, since the school closed one week earlier than was officially scheduled, data were only collected during the eight weeks following the start of the intervention. During the intervention period, 8 children in the yoghurt group and 10 children allocated to the placebo group were absent from school for three or more days per interval of two weeks. As these children missed the intake of the test product or placebo during the absence days, their data points were omitted from the study. Consequently, data were collected from a total of 178 children (per protocol population): 93 children in the yoghurt group and 85 children in the placebo group. Figure 1 shows a graphical representation of the study setup.

Data analysis

All data were collected through a specifically designed mobile software application (Kenga Mobile, OMNI-Tech Ltd), from which it was uploaded to a protected online platform with access by the researchers only. All children were registered in the app. The app contained three different 'forms', which could always be linked to a child: a form for parent questionnaires, a form for reporting incidences of diseases, and a form to report anthropometric measures, as described previously[7]. From the online data collection platform, data was exported to Microsoft Office Excel 2019 for analysis. To determine whether the difference in the incidence of RTIs and Skin Infections in the probiotic yoghurt group versus placebo over the intervention period of 57 days was significant, a Mann-Kendall test was performed at a 5% significance level using OriginPro version 2023b SR1 (OrginLab Corporation, Northampton, Massachusetts, USA). The *p*-values for the differences in primary and secondary study outcomes between the probiotic yoghurt and placebo group were calculated using a two-tailed Student's t-test for two samples assuming unequal variances (the Welch's t-test). The *p*-values for differences in study outcome measures during the baseline and intervention periods within groups were calculated using a two-tailed Student's t-test for two samples assuming equal variances.

Controlled interrupted time series analysis

To analyze the incidence of symptoms of RTI (rhinitis and cough) and skin infections, the average number of incidences per child per day per 100 children was considered as the unit of analysis. The incidences of rhinitis, cough, and skin infections were treated individually in a controlled, interrupted time-series experiment.

Analysis of anthropometric data

Anthropometric records were imported to WHO AnthroPlus software package provided by the World Health Organization for further analysis[22]. In this software package, measures of weight, height and Body Mass Index are assessed in reference to the WHO standard growth curves and are expressed as Z-scores. The formula for calculating the Z-score according to the WHO is Z-score = (X-m)/SD, in which X is the observed value (height, weight or BMI), m and SD are the mean and standard deviation value of the distribution corresponding to the reference population[23,24].

Urinary metabolite analysis and chemometric modelling of ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopic data

Urine samples were prepared for ^{1}H-NMR spectroscopy and data were acquired, as reported previously[25]. Acquired spectroscopic data were processed and imported into the SIMCA software package (version 13.0; Umetrics AB, Umeå, Sweden) for chemometric analysis. Supervised orthogonal projections to latent structures discriminant analysis (O-PLS-DA) models were calculated using one predictive component and two orthogonal components. The models were assessed based on variance explained (R^2Y) and predictive ability (Q^2Y) metrics[26]. From this multivariate statistical analysis, the following metabolites were identified as being of interest, and integrated from the ^{1}H-NMR spectroscopic data using the TopSpin 3.1 software package (Bruker Biospin, Rheinstetten, Germany). N-methylnicotinic acid (NMNA), formate, hippurate, phenylacetyl-glutamine, 4-cresol sulfate, citrate, aconitate, pyruvate, succinate, lactate, alanine, betaine, and creatine. The obtained integrals represent the concentrations of the metabolites in arbitrary units.

Fecal microbiota quantification

Quantification of the total microbial load and lactic acid bacteria (LAB) was conducted using flow cytometry-fluorescent in-situ hybridization (FC-FISH) with specifically designed probes. For total bacteria, the sequences were 5′ to 3′: GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT, and GCTGCCA CCCGTAGGTGT. For Lactic Acid Bacteria (*Lacticaseibacillus* and *Enterococcus*), the sequence 5′ to 3′ is GGTATTAGCAYCTGTTTCCA. Sample preparation, the design of the custom DNA oligonucleotides, labeling with Alexa488 or Alexa647 fluorescent dyes, flow cytometry analyses, quantification, and data interpretation was conducted according to a previously described method[27].

Immune-marker analysis in saliva

Secretory immunoglobulin A (sIgA) was measured in the collected saliva samples using sandwich ELISA as previously described[28]. Secretory leukocyte protease inhibitor (SLPI) was measured using an ELISA for recombinant human SLPI protein (Human SLPI Quantikine ELISA Kit DP100, R&D Systems), according to the manufacturer's protocol.

Survival analysis

Survival analysis was carried out to calculate the cumulative probability of children surviving (*i.e.,* without reporting an RTI or skin infection symptoms) a previous interval and making it to the next interval. Cumulative survival probabilities were estimated using the Kaplan-Meier method. A log-rank test was used to assess the statistical significance of the observed differences in survival curves[29]. The Cox proportional regression model was used to estimate hazard ratios. This multivariate method compares the 'survival experience' between two or more exposure groups while allowing for the simultaneous adjustment of confounding factors due to one or more covariates. The model was used to analyze how survival changes with varying covariate values such as age and sex[30].

Results

Socio-demographic characteristics and dietary indicators

The socio-demographic characteristics (including gender, average age, composition of household, toilet facility, source of water, source of drinking water, and more) of the two groups of 196 children participating in the study were comparable (Table 1). The dietary diversity scores (DDS) were determined three times: At baseline, halfway and at the end of the intervention period. DDS was nearly identical between the probiotic yoghurt and placebo groups. Moreover, the DDS indicated that, before the study started, 32% of the children in the probiotic yoghurt group and 46% of the children in the placebo group consumed milk products (Table 1). The increased consumption of dairy products during the intervention explains the slight increase in DDS halfway and towards the end of the study compared to the baseline values. At the household level, on an average day during the study period, 16% of the children in the probiotic yoghurt group and 14% of the children in the placebo group consumed fermented cereal porridge called bushera[31]. None of the children consumed fermented milk products at the household level.

Respiratory tract infection and skin infection rates

Following the exclusion of children who had missed more than three days per interval of two weeks to take the probiotic yoghurt or placebo product, data were analyzed from 93 children in the yoghurt group and 85 children in the placebo group. The total infection rate of RTI symptoms (scored by rhinitis and cough symptoms) and skin infection symptoms, as detected in both study arms, is shown in Figure 2A. For both arms, the infection rate increased during the weeks of baseline period, as well as during the first two weeks of the intervention period. The incidence of skin infections was higher than that of RTI. At baseline, the average number of recorded incidence of rhinitis, cough, or skin infections per day per 100 children was slightly, but not significantly, higher in the yoghurt group. Following the start of the intervention period, these infection rate values nearly doubled compared to baseline, without significant differences between the yoghurt and placebo groups (Table 2).

Notwithstanding the insignificant difference in the average number of recorded infections between the two groups at baseline, at the beginning of the intervention period, a higher incidence rate of rhinitis and cough was reported in the yoghurt group. Following the course of the intervention, the difference in the percentage of infected children between the yoghurt and placebo groups significantly declined over time, as concluded from the Mann-Kendall test (p=0.02) (Figure 2B). When comparing the percentage of children with skin infection symptoms between the groups, no significant trend was observed (Figure 2C).

Category	Sub-category	Placebo	Yoghurt
Gender	Male	47%	50%
	Female	53%	50%
Average age (y)		4.82	4.91
Gender head of household	Male	84%	83%
	Female	15%	16%
Average age household head		37.3	37.1
Average composition of household	Number of people	10.0	8.9
	Females < 5 years	1.8	1.6
	Males < 5 years	1.8	1.6
	Females 6–13 years	1.7	1.5
	Males 6–13 years	1.6	1.6
	Females 14–59 years	1.5	1.3
	Males 14–59 years	1.2	1.1
	Females > 60 years	0.2	0.3
	Males > 60 years	0.2	0.1
Education level head of household	No formal education	9%	7%
	Primary	26%	38%
	Secondary	39%	33%
	Tertiary	24%	21%
Toilet facility	Toilet	97%	96%
	No toilet	2%	3%
Water Source	Protected well or spring	26%	19%
	Borehole	5%	4%
	Open spring or well	16%	23%
	Surface water	2%	1%
	Rainwater	2%	1%
	Piped water	47%	51%
Preparation of drinking water	Boil water	87%	85%
	Stand and settle	1%	5%
	Purified	11%	9%
Diet Diversity Index	Baseline	5.2	4.9
	Midline	5.5	5.5
	Endline	5.7	5.4

Table 1. Sociodemographic characteristics of the study participants in the probiotic yoghurt ($N = 101$) and placebo groups ($N = 95$). Diet diversity index, and data about composition of household are expressed as means.

Diarrhea

The incidence of diarrhea was reported weekly by the parents or caregivers of the children. At baseline, only one value was recorded, which makes the comparison between the two groups unreliable. During the intervention period, the average incidence of diarrhea per 100 children per week showed a non-significant downward trend in both the groups (data not shown). On average, 2.28% of the children in the yoghurt group and 2.78% of the children in the placebo group had diarrhea. The differences between the groups were not statistically significant (Table 2).

Anthropometric indicators

The average age, weight, height, and anthropometric Z-scores—including height-for-age (HAZ), weight-for-age (WAZ), and body mass index-for-age (BAZ)—at baseline and study completion are summarized in Table 2. A HAZ ≤ −2 indicates stunted growth, a WAZ ≤ −2 indicates wasted, and a BAZ ≤ −2 indicates underweight. At baseline, children in both groups had average lengths and weights with Z-scores approximating the WHO standard, slightly below 0 on average. A small proportion of participants met the criteria for wasting, stunting, or underweight (Table 3). Comparison of BAZ scores at baseline and study completion revealed a reduction in 86% of children in the placebo group and 78% in the yoghurt group (supplemental file 1). By the end of the study, no significant differences in anthropometric parameters were observed between groups. However, within-group analyses showed a significant decrease in BAZ scores in both the yoghurt ($p = 0.02$) and placebo groups ($p = 0.005$, Table 4).

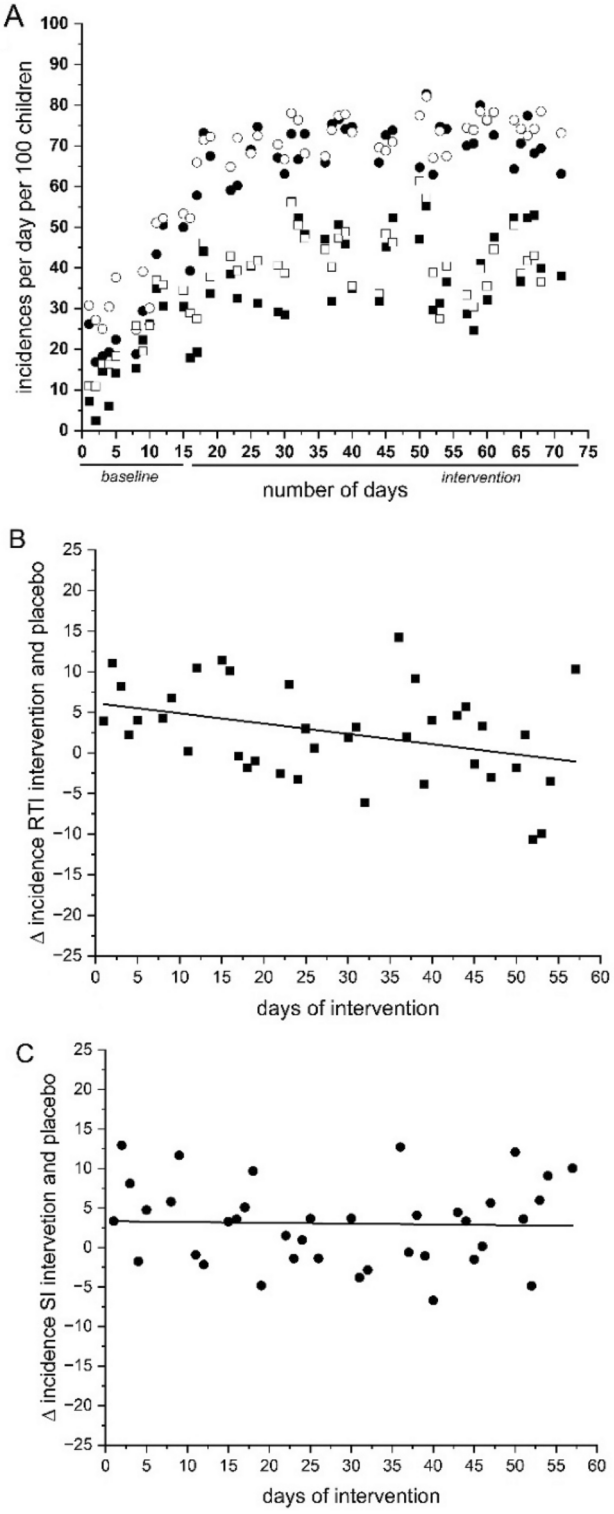

Fig. 2. **A**) Incidence of skin infection symptoms (SI) and respiratory tract infection symptoms (RTI) per day per 100 children. Open circles, SI probiotic intervention; closed circles, SI placebo; open squares, RTI probiotic intervention; closed squares, RTI placebo. **B**) Change in differences in the incidence of RTI between the probiotic yoghurt and placebo groups over time ($p = 0.02$). **C**) Change in differences in the incidence of SI between probiotic yoghurt and placebo over time.

Study outcomes			
Primary	**Placebo**	**Yoghurt**	***p*-value**
Respiratory tract infections (incidence/100 children/day)	39	42	0.23
Secondary			
Skin infections (incidence/100 children/day)	69	73	0.07
Diarrhoea (incidence/100 children/day)	2.78	2.28	0.76
Weight (kg)	17.8	18.0	0.76
Weight-for-Age (Z-score)	−0.25	−0.18	0.60
Body Mass Index-for-Age (Z-score)	−0.15	−0.07	0.55
Length (cm)	108	108	0.88
Height-for-Age (Z-score)	−0.24	−0.23	0.98
Absenteeism (absent days/100 children/week)	6.3	7.9	0.33
Urinary Hippurate (a.u.)	1.19	1.33	0.54
Urinary NMNA (a.u.)	0.04	0.05	0.60
Fecal Total Bacteria (10^8 CFU/gram)	5.22	7.35	0.11
Fecal Lactic Acid Bacteria (10^8 CFU/gram$^-$)	0.27	0.51	0.19
Salivary SlgA (ng/ml)	43	48	0.40
Salivary SLPI (ng/ml)	250	300	0.29

Table 2. Mean values of primary and secondary study outcomes assessed at the end of the study or over the full intervention period, as outlined in the experimental design (Fig. 1). Abbreviations:*a.u* Arbitrary Units, N-Methylnicotinic acid (NMNA), *CFU* Colony Forming Units, *SlgA* Secretory Immunoglobulin A, *SLPI* Secretory Leukocyte Protease Inhibitor. *p*-values were calculated using a two-tailed Student's t-test for two samples assuming unequal variances. p-values indicate the significance of differences between the placebo group ($N=85$) and the yoghurt group ($N=93$).

	Start of study			**End of study**		
	HAZ	**WAZ**	**BAZ**	**HAZ**	**WAZ**	**BAZ**
	Placebo Group Z-score < 0					
N (%)	46 (55)	49 (58)	29 (35)	53 (63)	51 (61)	49 (58)
	Placebo Group Z-score < −2					
N (%)	1 (1.2)	6 (7.2)	0 (0.0)	1 (1.2)	2 (2.4)	0 (0.0)
	Yoghurt Group Z-score < 0					
N (%)	44 (47)	54 (58)	35 (37)	46 (49)	51 (55)	49 (53)
	Yoghurt Group Z-score < −2					
N (%)	0 (0.0)	5 (6.0)	0 (0.0)	0 (0.0)	5 (6.0)	1 (1.2)

Table 3. Anthropometric outcomes expressed in Z-scores below 0 and below −2 as determined at the beginning and end of the study in placebo group ($N=85$) and yoghurt group ($N=93$). The percentage of the number of participants per group is indicated between brackets.

Absenteeism

After correction for long-term absences as required for reliable scoring of infection incidences (see Methods), absence from school, expressed as the number of average days of absence per 100 children per week during baseline was 4.3 and 6.5 for the yoghurt and placebo groups, respectively. During the intervention period, the average number of absence days per 100 children per week increased to 7.9 and 6.3 for the yoghurt and placebo groups, respectively. The reported values were not significantly different between the two groups.

Fecal bacterial load, urinary metabolites and immune biomarkers

Fecal microbial load measured at the beginning of the study as well as at the end of the study, was similar between both groups. However, the yoghurt group showed a significant increase in total bacterial count (p=0.04) and lactobacilli count (p=0.043) at the end of the study compared to the data collected at the beginning of the study (Figure 3). No significant differences in any of the measured urine metabolites were observed between the two study groups at baseline or during intervention. However, hippurate values significantly increased in the yoghurt group, but not in the placebo group, when comparing baseline data with data collected at the end of the study (Figure 4). The metabolite N-methylnicotinic acid (NMNA) significantly increased within each group when comparing baseline values with values collected at the end of the study in the yoghurt (p=0.01) and placebo (p=0.004) groups. When comparing the values of the salivary immune markers sIgA and SLPI recorded at the end of the study versus the beginning of the study, no significant differences were observed between the

	Placebo		p	Yoghurt		p
	Baseline	Endline		Baseline	Endline	
BAZ	0.20 (0.77)	−0.15 (0.81)	0.020	0.25 (0.92)	−0.07 (0.94)	0.005
Hippurate (a.u.)	1.18 (1.95)	1.19 (1.38)	0.957	0.88 (0.89)	1.33 (1.61)	0.020
NMNA (a.u.)	0.03 (0.03)	0.04 (0.04)	0.004	0.03 (0.04)	0.05 (0.03)	0.009
Bacteria (10^8 CFU g^{-1})	4.18 (4.31)	5.22 (5.66)	0.196	4.46 (7.09)	7.35 (10.0)	0.044
LAB (10^8 CFU g^{-1})	0.22 (0.46)	0.27 (0.52)	0.543	0.15 (0.28)	0.51 (1.51)	0.043
SLPI (μg ml^{-1})	0.36 (0.34)	0.25 (0.23)	0.025	0.48 (0.45)	0.30 (0.33)	0.005

Table 4. Differences in study outcome measures during the baseline and intervention periods within placebo group ($N = 85$) and yoghurt group ($N = 93$). Abbreviations: BAZ, BMI for age; Hippurate; N-methylnicotinic acid (NMNA) are metabolites in urine. Total bacteria and lactic acid bacteria (LAB) are expressed as concentrations in feces. Standard deviations are indicated between brackets. *p*-values were calculated using a two-tailed Student's t-test for two samples assuming the variances of the two groups are unequal. Statistically significant differences ($p<0.05$) are indicated.

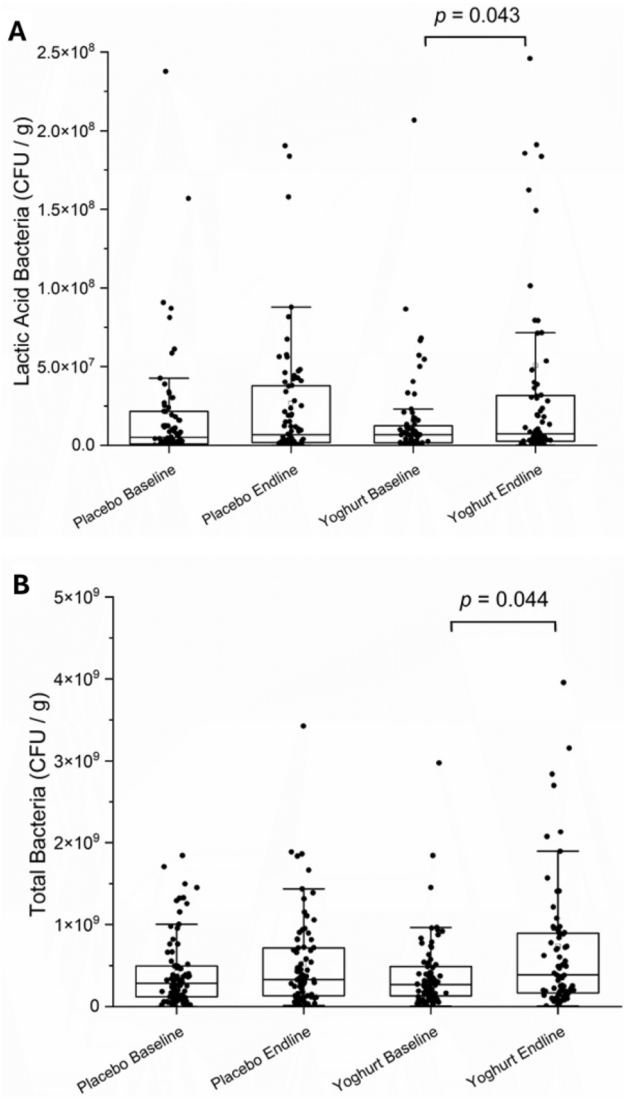

Fig. 3. Box plots for flow cytometric analysis of bacterial load in stool samples: **A**) lactic acid bacteria (CFU $gram^{-1}$) and **B**) total bacteria (CFU $gram^{-1}$). The *p*-values for differences in the bacterial loads were calculated using a two-tailed Student's t-test for two samples assuming equal variances (Placebo group $N = 85$, yoghurt group $N = 93$).

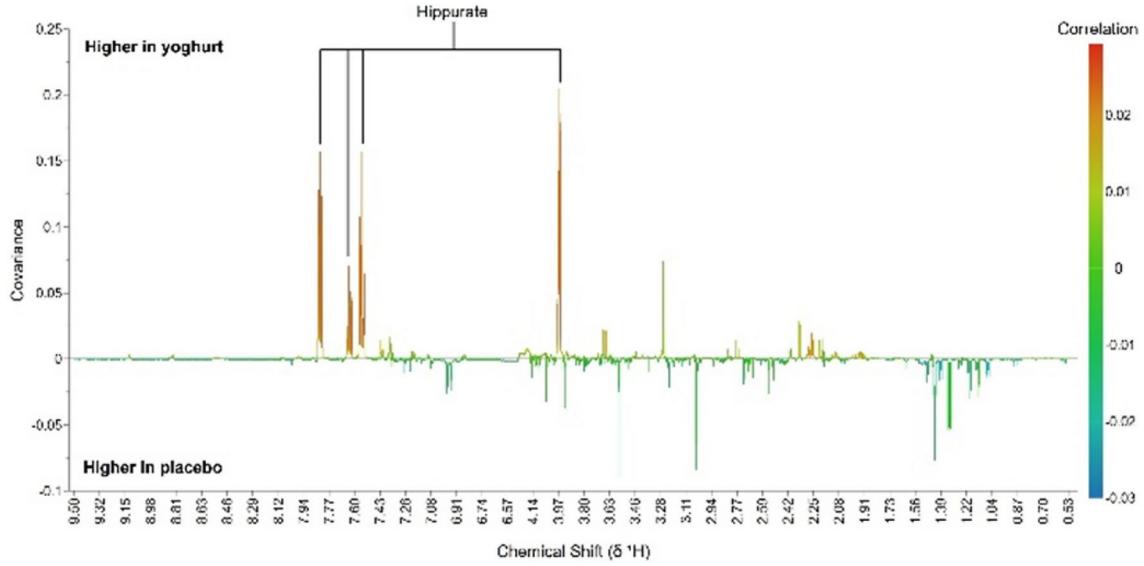

Fig. 4. Chemometric modelling of ^{1}H-NMR spectroscopic data acquired from urine samples. Differences in metabolites are indicated by direction (covariance) and color (correlation). Discriminatory metabolites were labelled (hippurate was positively associated with probiotic intake; placebo group $N = 85$, yoghurt group $N = 93$).

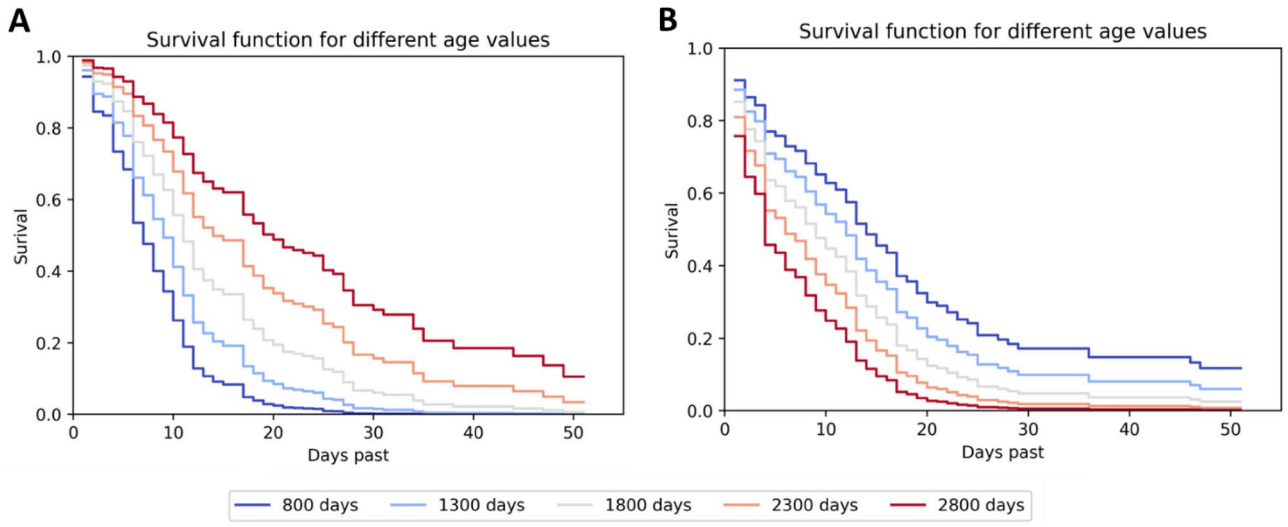

Fig. 5. Survival analysis showing the survival fraction of children categorized in different age groups for correlations between children's age and (**A**) incidence of respiratory tract infections and (**B**) incidence ofskin infections .

two groups. However, the SLPI values significantly decreased in the yoghurt group (p=0.005) and placebo group during the intervention (p=0.025) (Tables 2 and 4).

Survival analysis

Survival analysis was carried out to calculate the cumulative probability of children without indication of RTI or skin infection symptoms from one day to the following day. The subsequent application of a Cox proportional regression model showed that there was no significant difference in the probability of the occurrence of skin and RTI symptoms between males and females (p=0.42) or between the ages of the children (p=0.87). However, significant differences were observed when stratifying according to age, with children of lower age having a lower risk of developing skin infection symptoms (p<0.005) and children of higher age having a lower risk of developing RTI symptoms (p<0.005) (Figure 5).

Discussion

This randomized, double-blind, placebo-controlled study of probiotic yoghurt was conducted in a real-life setting among 196 children aged three to six years old in a school in Southwest Uganda. The study included respiratory tract infections (RTIs) as the primary health outcome and skin infection symptoms, together with objective health markers as secondary outcomes, including immune markers in saliva, metabolites in urine, and bacterial load in stool samples. A significant downward trend was found in the difference in the incidence of RTI symptoms over time between the probiotic yoghurt and placebo groups (p=0.02; Mann-Kendall test; Fig. 1B). The decreasing incidence rate of RTI symptoms in the probiotic yoghurt group relative to the placebo group during the intervention period suggests a positive effect of probiotic consumption on the incidence of RTI symptoms. This effect corresponds to the results of other studies and meta-analyses in which probiotic fermented dairy products, including food fermented with *Lacticaseibacillus rhamnosus* GG, were administered to children. [32–35] No significant differences were observed for any of the secondary outcomes between the yoghurt and placebo groups. However, intragroup analysis showed significant differences in hippurate levels in urine (p=0.02), load of lactic acid bacteria (p=0.04), and total bacterial loads (p=0.04) in stool between baseline and data collected at the end of the study in the yoghurt group. In addition, BAZ values, the urine metabolite N-methylnicotinic acid, and SLPI levels in saliva were significantly altered in both study groups.

During the baseline period, as well as during the first two weeks of the intervention, we observed an increase in RTI symptoms in both the probiotic yoghurt and placebo groups. More specifically, RTI symptoms were hardly observed when children returned from school holidays; however, the infection rate increased to 50%. A high incidence rate of tinea capitis observed during the study, with an average of approximately 70% in both groups, was also reported in our previously described study[7]. This level is much higher than that reported by other authors who have conducted studies on African children[36–39]. No clear explanation has been found for this exceptionally high overall incidence of skin infection symptoms. Similarly, as observed for RTI symptoms, the increase in skin infection symptoms during the first weeks of the study can be explained by the high contagiousness of most skin infections, including tinea capitis[40].

Several studies and meta-analyses have indicated that *Lacticaseibacillus rhamnosus* GG, to some extent, can reduce the formation of diarrhea, including rotavirus-induced diarrhea, travellers' diarrhea, and antibiotic-induced diarrhea[41–44]. However, the cause of diarrhea in the current study is unknown. In addition, data were not obtained through observation by the research team, but through reports of the parents as obtained by phone calls, which may not be reliable, as described in the following paragraph about the limitations of this study. Consequently, we cannot draw any conclusions regarding the relationship between the intervention and incidence of diarrhea in this study. Conclusive data on the effect of probiotics on diarrhea could be obtained in a more controlled setting (such as a hospital), where gastrointestinal symptoms can be defined and scored more accurately.

Variations in anthropometric indicators between the probiotic yoghurt and placebo groups were not statistically significant. However, we noticed a significant decrease in BMI between the start and end of the study in both groups (BAZ). This observation suggests that children who return from holidays at the start of the study spend more energy attending school compared to days spent at home. Several other studies that followed children for a longer period of time found positive effects on anthropometric indicators for milk consumption as well as probiotic consumption, as elaborated in our previous publication[7]. Conversely, these studies used larger quantities of dairy products, which might not be affordable in daily practice for the population targeted in the present study.

School-feeding has been shown to improve school attendance[7]. The current study did not show any positive effects of either probiotic yoghurt consumption or consumption of the placebo product on school attendance. A potential explanation could be that the absence rate was already quite low at the school in which the study was conducted. In addition, the school had already provided school feeding in the form of a maize porridge during the morning break time and a maize- and beans-based meal at lunchtime. The increase in absenteeism during the intervention period compared to the baseline period is explained by the fact that some children were sent home during the term when their parents had failed to (fully) pay the school fees for the term. These children were allowed to return to school only after paying the required fee. In addition, there were two days in which absenteeism was approximately 67%, as outlined in the limitations section.

Urinary levels of hippurate (the glycine conjugate of benzoic acid) were significantly higher in the probiotic yoghurt group than at baseline, but not in the placebo group. Known as a 'food intake biomarker (FIB)', hippurate is a normal constituent of the endogenous urinary metabolite profile and has long been associated with microbial degradation of specific dietary components[45]. More specifically, relatively high levels of hippurate in the urine have previously been associated with the intake of dairy products[46]. Levels of hippurate also increase with the consumption of benzoic acid, which is produced by the yoghurt starter culture of *Lacticaseibacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*[47]. This finding may explain the higher levels of hippurate observed in the probiotic yoghurt group. Human and experimental studies have shown that high urine hippurate concentration is associated with microbiome diversity, a general marker of metabolic health[48].Hence, we interpret these results as an indicator of the positive impact of the probiotic yogurt intervention.

A second significant finding was the increase in urinary NMNA levels in both study groups. Since increased levels of NMNA have been found to be a metabolic adaptation to reduce energy expenditure in a study in undernourished children[49], we speculate that the extra energy expenditure, as concluded from the reduction in the BAZ values in the probiotic yoghurt and placebo groups, could have led to a significant increase in NMNA in both study groups.

The bacterial loads in the stool of Ugandan children determined in our study by flow cytometry are relatively low with average values ranging from 0.15 to 4.5 x 10^8 cell counts per gram of stool. Previous studies on adult populations using FC-FISH methodology showed variations in microbial loads of two orders of magnitude,

between 10^8 and 10^{10} cell counts per gram of fecal material[50–52]. Few studies have enumerated bacteria from the stool of children using targeted quantification approaches such as FC-FISH, let alone school-age Ugandan children, as studied here. It is not clear at this point if the relatively low numbers found in this study can be attributed to this specific group of Ugandan children or if there are other factors contributing to a lower bacterial load. The increase in bacterial load during the intervention period versus baseline, as was observed only in the probiotic yoghurt group, is in agreement with the results from a previous study that reported an increase in *S. thermophilus* among yoghurt consumers in stool[53]. In addition, a recent study using an ex vivo model of the human intestinal microbial ecosystem showed that nutrient load acts as a driver of gut microbiota load[54].

Saliva is a non-invasive and stress-free tool for evaluating inflammatory markers. It is increasingly acknowledged that saliva can provide a useful matrix for the diagnosis of several diseases to study biomarkers of general health and stress, as referred to in a recent work[55]. Biomolecules in saliva can originate from different sources, either locally produced by the salivary glands or via diffusion or active transport from the blood[56]. SIgA is an antibody that functions primarily in the mucosal immune system. It serves as the first line of defence to protect the upper respiratory tract and the oral cavity.

In the present study, we did not observe any differences in the sIgA levels in either group. SIgA is studied frequently in children, but different analysis methods make the comparison of levels between different studies difficult. One study collected saliva samples from children aged one to six years enrolled in a sanitation trial in Maputo, Mozambique and characterized salivary SIgA concentrations using enzyme-linked immunosorbent assays. The median salivary SIgA concentration in this study population was 54 µg/mL (interquartile range (IQR): 34, 85 µg/mL), and SIgA levels were similar between children of different ages[57]. These levels are similar to those found in our study.

SLPI is a highly abundant protein in mucous secretions in the oral cavity and intestine. SLPI is known for its anti-inflammatory and antimicrobial role[58–60]. SLPI has mainly been studied in HIV-1 infection and oral health studies in adults, and values for salivary SLPI in children are scarce. One study in 188 infants (Kenia) at birth and at ages one, three, and six months investigates SLPI levels in saliva and found that the median salivary SLPI concentrations were higher at birth than at 6 months (341 vs. 219 ng/mL)[61]. In our study, SLPI levels were significantly decreased in both groups compared to the baseline results at the end of the study. During the course of the intervention, several effects that were potentially associated with the regulation of salivary immune biomarkers could have occurred simultaneously, and the interpretation of the observed results was difficult. For instance, children in the yoghurt group consumed the probiotic *Lacticaseibacillus rhamnosus* strain with assumed immune-enhancing effects. At the same time, the children in this group as well as the placebo group showed relatively high levels of RTI and skin infection symptoms during the entire study period.

This study was designed as part of an ongoing developmental program that introduced milk and yoghurt into schools, as previously outlined[7]. The study minimized the distortion of real-life school practices with the aim of analyzing the effects of the intervention in a representative school setting, rather than extracting results from an adapted experimental setup, which may not be translational to real-life practice. Consumption of locally produced probiotic yoghurt by children continued after the study as part of a school yoghurt program. Furthermore, the probiotic yoghurt and placebo groups had similar socio-demographic characteristics. However, the extent to which the potential health benefits of daily administration of probiotic yoghurt would depend on the socio-demographic parameters of the study participants.

The limitations of the study include the increase in both RTI and skin infection symptoms during the baseline and the first two weeks of the intervention period. We attribute this observation to the occurrence of cross-infection among children as they returned to school at the beginning of the term. This hypothesis is supported by reported risk ratios of 1.5 – 3.0 for contracting infectious diseases such as common cold for children attending day care centres compared to children who are kept at home[32,62,63]. This is an important finding that may guide the design of further research on related topics, as this cross-infection effect distorts the results and subsequent conclusions of health intervention studies. We speculate that in the present setting, in which children receiving probiotic yoghurt and placebo were in the same class, infection pressure from the placebo group in the probiotic yoghurt group remained present during the entire study period. We hypothesized that the probiotic yoghurt intervention would have led to a faster reduction of infections if the intervention and placebo groups were allocated to different school classes, as we assumed a faster rate of cross-infections for children in the same class compared to children in separate classes. Indeed, our previous study[7],designed with control and intervention groups in separate classes, showed a stronger effect of probiotic yoghurt intake in particular with regard to the reduction of skin diseases. In fact, the desired situation of having one class where all the children take probiotic yoghurt is a daily practice in the probiotic yoghurt program that has been implemented in Southwestern Uganda. In the current study, children were only followed during a single school period of 12 weeks, of which the first three weeks were supposed to be used for baseline data collection. However, in practice, the study period was further shortened, as during the first and the last week of the study, it was not possible to collect data because only a small number of children attended school, and because a large number of children had already gone home for holidays.

Previous studies on the effect of *L. rhamnosus* on upper RTI symptoms in healthy children reported significant reductions in RTI symptoms after a minimum of three months of intervention[32,34,64–66]. Although the exact mechanism is not fully known, *L. rhamnosus* GG has been reported to attenuate the incidence of common cold and skin diseases through stimulation of the humoral, cellular and non-specific immunity system of the host[32,63,67]. Stimulation of adaptive immunity takes a couple of weeks. The duration of the lag phase of immune system stimulation by probiotics is not known but may be longer and differ between individuals. Consequently, the intervention period of eight weeks in the present study might have been too short to assess the effect of probiotic yogurt consumption.

The effects of confounding factors cannot be excluded. In addition to a non-significant difference in baseline values between the placebo and probiotic yoghurt groups, we observed a growing level of cross-infection during the baseline and first weeks of the intervention for RTI and skin infection symptoms. Before drawing definitive conclusions on the health impact of probiotic yoghurt consumption as part of school feeding programs, we recommend follow-up studies over a longer period, with additional controls and a more uniform background, such as in the setting of a boarding school. Further recommendations resulting from the present study suggest pre-administration of probiotic yoghurt prior to the start of the school term and physical separation of the randomized groups in different classes with the aim of avoiding potential cross-infection and infection pressure by children suffering from skin- and common cold-related infection symptoms.

Data availability

All data supporting the findings of this study are available within the paper and supplemental file 1.

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Author contributions

Conceptualization: WS, NW, and RK. Writing- Original draft preparation: WS, NW, and RK. Data curation: WS, NW, and RK. Urinary metabolite analysis and bacterial load analysis of faeces: CD, AW. Immunomarker analysis of saliva: JG. Monitoring of health outcomes at pre-primary schools: NW and EG. Coordination of probiotic yoghurt and placebo product production and facilitation of the school feeding program: JT. Supervision: WS, RK. Writing- Review and Editing of the final manuscript: WS and RK. All authors have read and approved the final manuscript.

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Declarations

Competing interests

RK and WS are co-founders of the Yoba for Life Foundation (2009), a non-profit organization accredited by the Dutch Tax Authorities as a Public Benevolent Institution (PBI), which aims to promote local production and consumption of fermented products in Africa. Nieke Westerik, Johnbosco Tumuhimbise, and Els Gregorowitsch are employees of the Yoba for Life Foundation in Uganda. The other authors declare no conflicts of interests.

Ethical approval

This trial has been approved by the MUST Research Ethics Committee (MUST-REC). Approval Number: 01/09-18. Board Affiliation: Mbarara University of Science and Technology, Mbarara, Uganda. This trial has been registered at ClinicalTrials. gov (NCT04144491).

Additional information

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