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Randomized control trials

Cow's milk and rice fermented with *Lactobacillus paracasei* CBA L74 prevent infectious diseases in children: A randomized controlled trial

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SUMMARY

Background & aim: Fermented foods have been proposed for the prevention of infectious diseases. We evaluated the efficacy of fermented foods in reducing common infectious diseases (CIDs) in children attending daycare.

Methods: Prospective randomized, double-blind, placebo-controlled trial (registered under Clinical Trials.gov identifier NCT01909128) on healthy children (aged 12–48 months) consuming daily cow's milk (group A) or rice (group B) fermented with *Lactobacillus paracasei* CBA L74, or placebo (group C) for three months during the winter season. The main study outcome was the proportion of children who experienced at least one CID. All CIDs were diagnosed by family pediatricians. Fecal concentrations of innate (α- and β-defensins and cathelicidin LL-37) and acquired immunity biomarkers (secretory IgA) were also evaluated.

Results: 377 children (193 males, 51%) with a mean (SD) age of 32 (10) months completed the study: 137 in group A, 118 in group B and 122 in group C. Intention-to-treat analysis showed that the proportion of children who experienced at least one CID was lower in group A (51.8%) and B (65.9%) compared to group C (80.3%). Per-protocol analysis showed that the proportion of children presenting upper respiratory tract infections was lower in group A (48.2%) and group B (58.5%) compared with group C (70.5%). The proportion of children presenting acute gastroenteritis was also lower in group A (13.1%) and group B (19.5%) compared with group C (31.1%). A net increase of all fecal biomarkers of innate and acquired immunity was observed for groups A and B compared to group C. Moreover, there was a negative association between fecal biomarkers and the occurrence of CID.

Conclusion: Dietary supplementation with cow's milk or rice fermented with *L. paracasei* CBA L74 prevents CIDs in children attending daycare possibly by means of a stimulation of innate and acquired immunity.

1. Introduction

Daycare centers and schools are ideal places for the occurrence and transmission of common infectious diseases (CIDs) affecting respiratory and gastrointestinal tract in young children, often resulting in many missed days of both daycare and parental work [1]. Young children attending daycare centers and schools have a 1.5–3.0 times higher risk of respiratory and gastrointestinal tract

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Abbreviations

CIDs	common infectious diseases
AGE	acute gastroenteritis
URTI	upper respiratory tract infections
FPs	family pediatricians
HNP	1-3: α-defensin
HBD-2	β-defensin
LL-37	cathelicidin
sIgA	secretory immunoglobulin

infections than children cared for at home or in small family care groups [1]. These subjects have been shown to have more outpatient doctor visits, emergency room visits, and increased usage of prescription medicines than children not in daycare [2]. Daycare-related infectious illnesses have been estimated to cost $1.8 billion per year in the United States [3].

The prevention of infections in daycare is therefore of major importance. An option is to use fermented foods with probiotics [4]. The fermentation process provides a gain in these food products in terms of benefits for health, which makes these products useful strategy against pediatric infections [4]. The efficacy of these fermented foods is believed to be strain-specific and dose-dependent [4]. For these reasons, it is of fundamental importance to test each product in clinical trials.

The aim of this double-blind, randomized, placebo-controlled trial was to test the preventive effect of fermented products with *Lactobacillus paracasei* CBA L74 (i.e., fermented milk and rice) against CIDs in children attending daycare or preschool. We also tested whether these fermented foods are able to stimulate innate (α- and β-defensins and cathelicidin LL-37) and acquired (secretory immunoglobulin A) immunity factors.

2. Patients and methods

2.1. Study design

A prospective randomized, double-blind, placebo-controlled trial was conducted from January to March 2012 in collaboration with family pediatricians (FPs), who care for children up to 14 years of age in the Italian Public Health System. The study protocol was illustrated to and discussed with FPs during two meetings.

2.2. Ethics

The study was approved by the Ethics Committee of the University of Naples "Federico II" and was registered in the Clinical Trials Protocol Registration System (ClinicalTrials.gov) with the identifier NCT01909128.

2.3. Study subjects

Consecutive healthy children (12–48 months of age) attending daycare or preschool at least five days a week, were invited to participate to the study. Anamnestic, demographic and clinical data, including vaccination status, were collected by the FPs and reported in a specific clinical chart. The exclusion criteria were: age ≤12 months or ≥48 months, concomitant chronic systemic diseases, congenital cardiac defects, gastrointestinal or urinary or respiratory tract surgery, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, cystic fibrosis, metabolic diseases, history of suspected or challenge-proved food allergy, lactose intolerance, malignancy, chronic pulmonary diseases, malformations of gastrointestinal or urinary or respiratory tract, severe malnutrition (z score for weight-for-height <3 standard deviation scores), and use of pre/pro/synbiotics, antibiotics or immune stimulating products in the 2 weeks before the enrollment.

2.4. Intervention

The investigators were blinded to the treatment at all times, i.e. allocation, intervention, laboratory analysis and statistical analysis. The study subjects were distributed into three groups (A, B and C) according to a computer-generated randomization list. The FPs assigned for each child the next available number on entry into the trial. The FPs, parents and children were not aware of the dietary treatment assigned. Subjects were supplemented daily for 3 months with a dietary product deriving from cow's milk (group A) or rice fermentation (group B) with *L. paracasei* CBA L74, or placebo (group C).

In Table 1 is reported the composition of the study dietary products. They were provided in powder by Heinz Italia SpA, Latina, Italy, an affiliate of H.J. Heinz Company, Pittsburgh, PA, USA. The fermented milk was prepared from skim milk fermented by *L. paracasei* CBA L74. The fermentation was started in the presence of 10^6 bacteria, reaching 5.9×10^9 colony-forming units/g after a 15-h incubation at 37 °C. After heating at 85 °C for 20 s in order to inactivate the live bacteria, the formula was spray-dried. Thus, the final fermented milk powder contained only bacterial bodies and fermentation products and no living microorganisms. The fermented rice product was obtained using the same procedure. The placebo consisted of maltodextrins with similar energy content of fermented milk and rice products. Study products were provided in tins containing 400 g of powder, and the packaging was similar. Study products were stored at room temperature and in a dry environment.

The FPs instructed parents about the daily amount of the assigned study product and the method of preparation. All subjects received 7 g/day of study products diluted in a maximum 150 ml of cow's milk or water. After dilution, the look and the taste were the same for all study products. Parents were encouraged to contact the FP if necessary and to maintain the habitual diet of the child, but to exclude prebiotics, probiotics, synbiotics and immune stimulating products during the 3-month study period.

During episodes of acute gastroenteritis (AGE), children were instructed to continue the assigned study product.

An independent clinical trial monitor, blinded to the treatment assignment, was involved in the research. Study monitoring included on-site visits and telephone communications with FPs, to ensure that the investigation was conducted according to the protocol. The clinical trial monitor collected clinical forms, ensured compliance with the clinical trial protocol, reviewed the clinical forms for completeness, clarity, and consistency, and communicated with the clinical research coordinators before the final analysis.

2.5. Study outcomes

The primary outcome of the trial was the proportion of children experiencing at least one episode of CID. The secondary outcomes

Table 1
Composition of the study dietary products.

Value for 100 g of product	Fermented milk	Fermented rice	Placebo
Energy, kcal	367	397	388
Proteins, g	24.0	7.8	0
Carbohydrates, g	66.4	88.9	97
Fats, g	0.6	1.1	0
Lactobacillus paracasei CBA L74, CFU[a]	5.9×10^{11}	5.9×10^{11}	–

[a] Killed bacteria.

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were the proportion of children with recurrent CID (i.e. ≥3 episodes), total number of CIDs, use of medications (antipyretics, antibiotics, or steroids), emergency department visits, pediatric visits by the FP and hospitalizations. Study groups were also compared for fecal levels of α- and β-defensins, cathelicidin (LL-37), secretory immunoglobulin A (sIgA) at enrollment and after 3 months of intervention. The occurrence of adverse events were also recorded.

2.6. Estimate of sample size and randomization

We calculated that 118 children per group were needed to detect a change in the occurrence of at least one episode of CID from 80% in group C to 60% in groups and A and B with a power of 0.92 at an alpha level of 0.05 (Pearson's Chi-square, two-tailed test). The pre-planned comparisons of interest were therefore A vs. C and B vs. C. We randomly assigned treatments with a computer-generated randomization sequence using block sizes of 36 subjects per FP.

2.7. Data collection

A diary was given to the parents by FPs, with instructions to report daily: fever, gastrointestinal or respiratory symptoms, use of drugs, emergency department visits, hospitalizations, possible adverse events, and assumption of the study products. For all the children a visit was planned by the FP at 30, 60 and 90 days (final visit), and whenever it was necessary because infectious diseases or other morbidities. During these visits, the general clinical conditions of the children were evaluated, the study products were provided to the parents for the next 4 weeks, and the product tins were collected. All infectious diseases were recorded by FPs in a specific study collection form. The diagnosis of infectious diseases was made by the FPs based on the evaluation of specific symptoms according to standardized definitions. The diagnosis of AGE was identified by the presence of ≥3 episodes of soft/liquid feces in 24 h with or without fever or vomiting [5]. The presence of upper respiratory tract infections (URTI) was defined as the occurrence of ≥1 respiratory symptom(s) (runny nose, cough, sore throat, aphony, shortness of breath, otalgia, otorrhea, extroversion of tympanic membrane with or without hyperemia) in the absence or presence of ≥1 systemic symptom(s) (fever, headache, restless, myalgia, irritability). These symptoms were evaluated by the FP who made a final diagnosis of rhinitis, tracheitis, pharyngitis, laryngitis or otitis media [6–8].

Compliance was defined as the consumption of at least 80% of the assigned treatment during the study and was evaluated by counting and weighing the returned tins and by the notes on the diary compiled by parents.

2.8. Assessment of immunological parameters

At the enrollment and at the end of the trial a stool sample (3 g) was obtained from all study subjects. Samples were immediately frozen (−20 °C), brought frozen to the laboratory and stored at −80 °C. Fecal α-defensin (HNP 1-3), β-defensin 2 (HBD-2), LL-37, and sIgA were measured from the supernatants of fecal homogenates. Briefly, homogenates were centrifuged (13.000 × g, 10 min), and the supernatants were collected. For LL-37 measurement, the samples were extracted with 60% acetonitrile in 1% aqueous trifluoroacetic acid (TFA) and then extracted overnight at 4 °C as described previously [9]. The extracts were centrifuged and supernatants stored at −20 °C. Concentrations of HBD-2, HNP 1-3, LL-37 and sIgA were measured by ELISA with β-defensin 2 (Human) ELISA kit (Phoenix Pharmaceuticals, Inc., USA), HNP 1-3 human (Hycult biotechnology, Uden, Netherlands), sIgA Indirect Enzyme Immunoassay Kit (Salimetrics LLC, USA) and LL-37, Human, ELISA (Hycult biotechnology, Uden, the Netherlands) respectively, according to the manufacturer's instructions. The results were expressed as ng/g for α-defensin, β-defensin, and LL-37 and as μg/g of supernatants for sIgA.

2.9. Statistical analysis

Descriptive statistics are reported as means and standard deviations for continuous variables and as numbers and proportions for dichotomous variables. The main outcome was the occurrence of at least one episode of CID during the 3-month study period. Such outcome was evaluated using intention to treat (ITT) analysis assigning the worst event, i.e. the occurrence of one CID, to the 14 children with missing data. Binomial regression was used to calculate the absolute risk difference (ARD) for the occurrence of at least one CID in group A *vs.* group C and in group B *vs.* group C. As CID is a composite outcome, we performed also a per-protocol (PPA) analysis on its components, i.e. URTI and AGE. A pre-specified secondary outcome was the incidence rate of CID in group A *vs.* group C and in group B *vs.* group C. Such outcome was estimated using Poisson regression under PPA.

Other pre-specified secondary outcomes were the changes of α-defensin, β-defensin, LL-37 and sIgA in group A *vs.* group C and in group B *vs.* group C. Such changes were analyzed using random-effect linear regression. The response variable was the immunological marker (continuous) and the predictors were the baseline value of the response variable (continuous), time (discrete, 0 = baseline; 1 = 3 months), treatment (discrete, 0 = group C; 1 = group A; 2 = group B), and a treatment × time (discrete × discrete) interaction. The treatment × time interaction gives a measure of the change in the immunological marker for the groups A and B *vs.* group C at 3 months *vs.* baseline. Immunological markers were $\log_e$-transformed to reduce skewness and ensure homoscedasticity of residuals.

Binary logistic regression analysis was used to evaluate the influence of sex, breastfeeding, age at enrollment, weight at enrollment, age at schooling, passive smoking, presence of sibling and treatment assignment on the primary outcome of the study. The regression coefficients obtained from logistic regression analysis were exponentiated and reported as odds ratios.

Spearman rank correlation coefficient (rho) was used to evaluate associations between fecal biomarkers and the total number of CID.

Statistical analysis was performed using SPSS 19.0 for Windows and Stata 14.1 (Stata Corporation, College Station, TX, USA).

3. Results

3.1. Clinical outcomes

The flow of children during the study is reported in Fig. 1. At baseline, the main features of the study groups were similar (Table 2). All children were from families of middle socioeconomic status. All children were not febrile at inclusion and none was suffering from respiratory tract or gastrointestinal symptoms. The vaccination status was identical among the three groups. No child had received anti-Rotavirus or anti-influenza vaccine. These vaccines are not usually included in the vaccination schedule of Italian healthy children.

The interventions were well accepted by the children and the overall compliance to them was good. Only 4 children in group A, 5 in group B and 5 in group C were lost to follow-up. No adverse events related to the consumption of the active or placebo products occurred during the study. No differences were detected in the daily intake of the active and placebo products between the study groups. No significant changes were observed for body weight and height in groups A, B and C during the study (data not shown),

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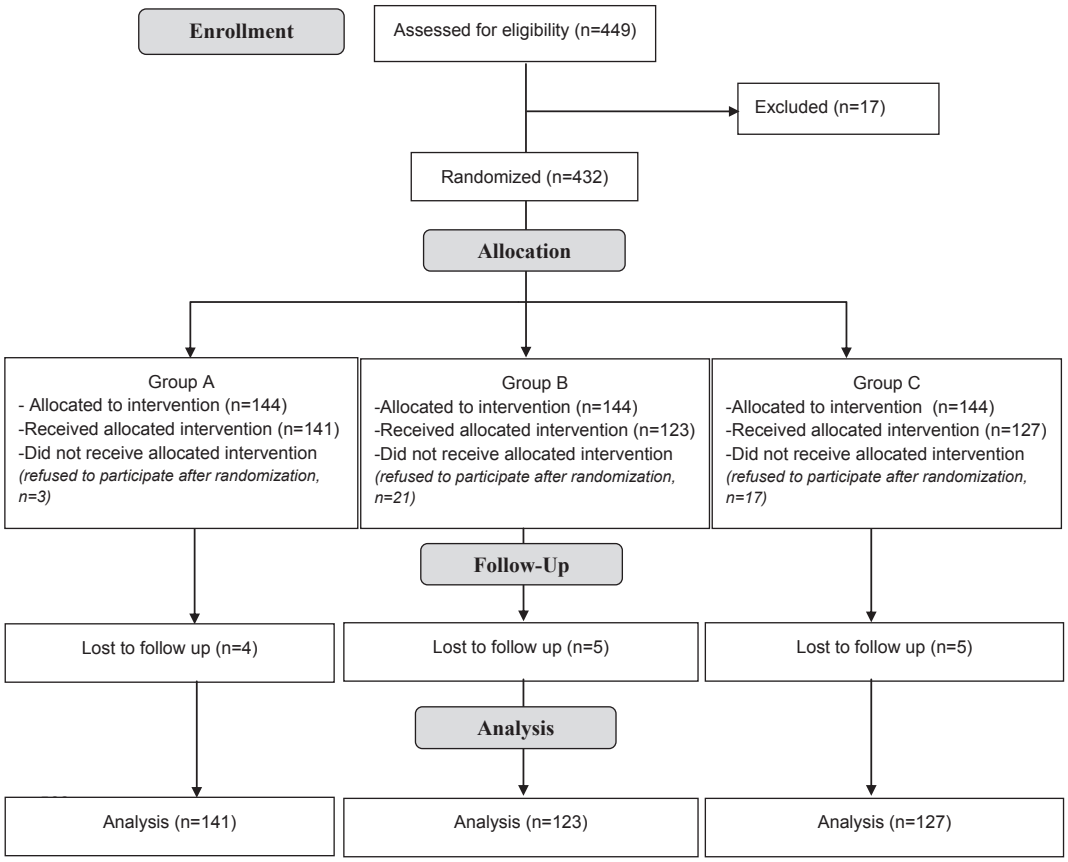

Fig. 1. The flow of children through the study.

indicating that the consumption of the study formula was safe, at least in the short term.

Intention-to-treat analysis showed that during the study period 256 out of the 391 (65.5%) children experienced at least one episode of CID. The proportion of children presenting at least one episode of CID was significantly lower in groups A and B compared with group C (Fig. 2). The absolute risk difference of the occurrence of at least one CID was −29% (95% CI: −39% to −18%, $p < 0.001$) for group A vs. group C and −14% (95% CI: −25% to −4%, $p < 0.01$) for group B vs. group C. These absolute risk reductions (ARRs) correspond to a number of children to treat (NNT) of 3 (95% CI 3 to 6) for group A and 7 (95%CI 4 to 25) for group B vs. group C. Per-protocol-analysis showed that the proportion of children presenting at least one episode of AGE was significantly lower in groups A and B compared with group C. The absolute risk difference of the occurrence of at least one episode of AGE was −18% (95% CI: −28% to −8%, $p < 0.001$) in group A and −12% (95% CI: −23% to −1%, $p < 0.05$) in group B compared to group C. Similar findings were observed regarding the proportion of children presenting at least one episode of URTI, that was significantly lower in group A vs. group C but not in group B vs. group C. The absolute risk difference of the occurrence of at least one episode of URTI was −22% (95% CI: −34% to −11%, $p < 0.001$) in group A and −12% (95% CI: −24% to −0%, $p = 0.05$) in group B compared to group C (Fig. 3).

The incidence rate ratio (IRR) calculated using Poisson regression under PPA was 0.36 (95%CI 0.29 to 0.44, $p < 0.001$) in group A and 0.61 (95%CI 0.51 to 0.73, $p < 0.001$) in group B vs. group C.

The proportion of subjects who experienced at least one episode of rhinitis, otitis, pharyngitis, laryngitis, tracheitis, or AGE and the total number of episodes were significantly lower in children in groups A and B, compared to subjects in group C either after Bonferroni correction (Table 3).

During the study period, the odds of receiving at least one medication course was significantly lower in groups A (OR 0.26, 95%CI 0.15 to 0.43) and B (OR 0.55, 95%CI 0.33 to 0.91) compared with group C (Fig. 4). During the study period, the FPs recorded 142 visits in group A, 208 in group B and 353 in group C. No child required emergency visit or hospitalization.

Among the compliant children, the proportion of those with recurrent common infections, i.e. ≥3 episodes *vs.* 1 to 2 infections, was 37% in group C, 10% in group A ($p < 0.001$ vs. group C, Wald test, binomial regression) and 22% in group B ($p = 0.07$ vs. group C, Wald test of binomial regression).

Binary logistic regression analysis was performed to evaluate the influence of the following variables on the primary outcome: sex, breastfeeding, age at enrollment, weight at enrollment, age at

Table 2
Main features of the study population at enrollment.

	group A	group B	group C
	n = 141	*n = 123*	*n = 127*
Male, n (%)	72 (51.1)	64 (52)	65 (51.2)
Age, months (±SD)	32 (10)	31 (11)	34 (9)
Weight, kg (±SD)	14.6 (2.8)	14.5 (2.7)	14.8 (2.9)
Height, cm (±SD)	93.3 (9)	92.7 (9)	94.3 (7.2)
Breastfeeding, n (%)	99 (70.2)	88 (71.5)	97 (76.4)
Duration of breastfeeding, months (±SD)	7.6 (6.1)	6.2 (4)	6.4 (5.1)
Age at schooling, months (±SD)	13.4 (2.4)	12.9 (2.2)	12.8 (2.3)
Siblings, n (%)	108 (76.6)	96 (78)	100 (78.7)
N. of siblings, (±SD)	1.30 (0.6)	1.4 (0.6)	1.4 (0.6)
Passive smoking, n (%)	65 (46.1)	56 (45.5)	59 (46.5)

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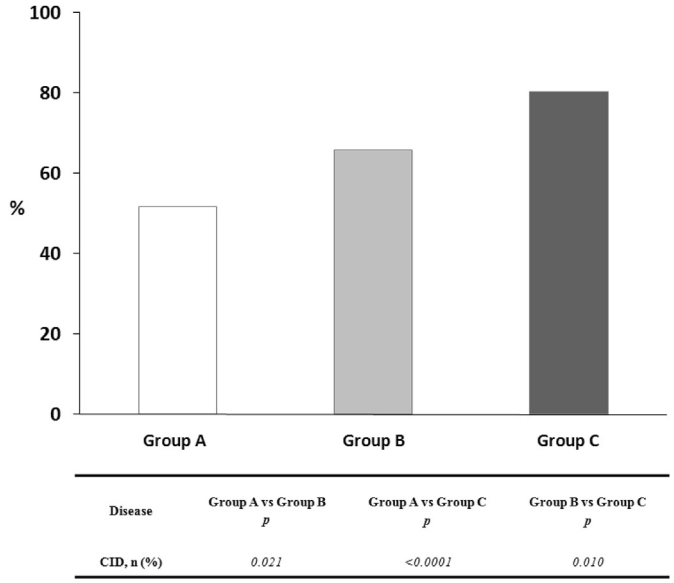


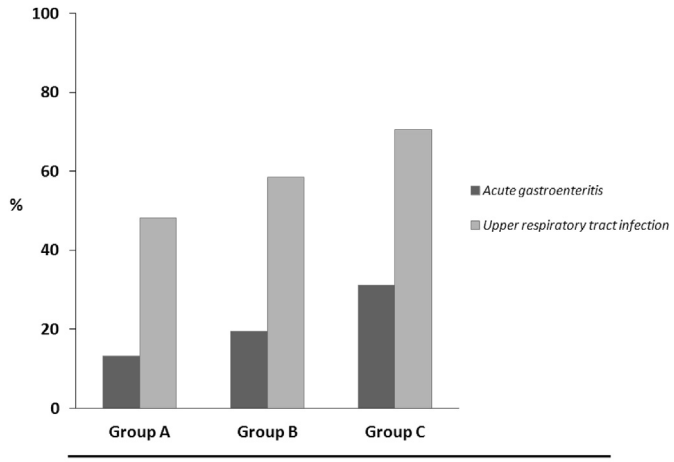
Disease	Group A vs Group B p	Group A vs Group C p	Group B vs Group C p
CID, n (%)	0.021	<0.0001	0.010

Fig. 2. The rate of children presenting at least one common infectious disease (ITT analysis) during the study period.

schooling, passive smoking, presence of siblings and treatment assignment.

The only variable positively associated with the main outcome was the presence of siblings (OR = 6.8, 95%CI 3.8 to 11.9, p < 0.001), while the active treatment (fermented milk or fermented rice) was the only variable negatively associated with the main outcome (OR = 0.28, 95%CI 0.16 to 0.48, p < 0.001).

Comparing groups A and C, the only variable positively associated with the main outcome was the presence of siblings (OR = 6.6, 95%CI 3.3 to 13.5, p < 0.001), while the active treatment was the only variable negatively associated with the main outcome (OR 0.19, 95%CI 0.11 to 0.37, p < 0.001). Likewise, comparing groups B and C the only variable positively associated with the main outcome was the presence of siblings (OR = 4.8, 95%CI 2.4 to 9.5, p < 0.001), while active treatment was the only variable negatively associated with the main outcome (OR = 0.65, 95%CI 0.48 to 0.89, p = 0.006).



Disease	Group A vs Group B p	Group A vs Group C p	Group B vs Group C p
Acute gastroenteritis, n (%)	0.169	<0.0001	0.038
Upper respiratory tract infection, n (%)	0.100	<0.0001	0.032

Fig. 3. The rate of children presenting at least one episode of acute gastroenteritis or at least one episode of upper respiratory tract infection (PP analysis) during the study period.

3.2. Innate and acquired immunity biomarkers

Figure 5 plots the changes in fecal $\log_e\alpha$-defensin, $\log_e\beta$-defensin, $\log_e$LL-37 and $\log_e$sIgA during the study. Net changes in $\log_e\alpha$-defensin ($p < 0.001$), $\log_e\beta$-defensin ($p < 0.01$), $\log_e$LL-37 ($p < 0.001$) and $\log_e$sIgA ($p < 0.001$) were seen at 3 months *vs.* baseline for group A *vs.* group C. Likewise, net changes in $\log_e\alpha$-defensin ($p < 0.001$), $\log_e\beta$-defensin ($p < 0.001$), $\log_e$LL-37 ($p < 0.001$) and $\log_e$sIgA ($p < 0.001$) were seen at 3 months *vs.* baseline for group B *vs.* group C.

A negative association was also detected between the change of α-defensin (rho = −0.362, p = 0.004), β-defensin (rho = −0.256, p = 0.047), LL-37 (rho = −0.296, p = 0.012) and sIgA (rho = −0.356, p = 0.001) and the total number of CIDs.

4. Discussion

This RCT provides evidence that a daily supplement with dietary products deriving from cow's milk or rice fermentation with *L. paracasei* CBA L74 protects the children attending daycare or preschool from contracting CIDs. The overall number of children presenting at least one episode of CID, the total number of CIDs and their number per child revealed statistically significant differences in favor of the treated groups. This protective effect was accompanied by a reduction of drugs use. The two active dietary products were well accepted by the children and safe, as demonstrated by the low drop-out rate together with the high level of adherence, and the absence of adverse events observed during the trial.

As for similar RCTs [3,10–12], our inability to evaluate infectious episode etiologies in enrolled children represents a major limit of the study. However, the strengths of our investigation include adequate randomization and power to test the hypothesis, the use of a double-blind design, comprehensive follow-up strategy, all of which minimize the risk of bias.

The critical role of pediatric nutrition on immune system is widely recognized, and we provided data on possible mechanisms of action elicited by these dietary products in protecting the children from CIDs. The same *L. paracasei* CBA L74-based fermented cow's milk product showed immunoregulatory activities on human dendritic cells and protective effects against *Salmonella thyphimurium* in the animal model [13]. Here, we observed an immunostimulatory effect consisting in a significant increase in innate and acquired immunity peptides production. Innate immunity peptides, produced by epithelial cells, paneth cells, neutrophils and macrophages, act as endogenous antimicrobial substances in the defence against a broad range of microbes (bacteria, fungi, and protozoa) and viruses [14]. In addition to their antimicrobial role, they have been shown to display several regulatory activity on innate and adaptative immune system. They have been described to regulate the activity of T cells, dendritic cells, macrophages, monocytes and neutrophils [15]. In parallel, the production of sIgA, a relevant component of acquired immunity, resulted positively affected by the two fermented dietary products. Similar results have been reported in several studies investigating probiotics or synbiotics [3,10–12]. In the present study killed probiotic was used. This suggest that the viability of the bacteria is not a fundamental factor to exert an effect on immune system, as also suggested by others [16,17]. Secretory IgA not only play a pivotal role in local immunity conferring the first

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Table 3
Common infectious diseases observed during the study period.

Disease	Group A	Group B	Group C	Group A vs Group B	Group A vs Group C	Group B vs Group C
				P	*P*	*P*
Acute gastroenteritis, n (%) [number of episodes]	18 (13.1) [21]	23 (19.5) [26]	38 (31.1) [47]	*0.169*	*<0.0001*[a]	*0.038*
Rhinitis, n (%) [number of episodes]	19 (13.9) [23]	31 (26.3) [47]	35 (28.7) [46]	*0.013*	*0.003*[a]	*0.675*
Otitis, n (%) [number of episodes]	3 (2.2) [3]	5 (4.2) [5]	18 (14.8) [31]	*0.477*	*<0.0001*[a]	*0.006*[a]
Pharyngitis, n (%) [number of episodes]	21 (15.3) [28]	41 (34.7) [57]	53 (43.4) [84]	*<0.0001*[a]	*<0.0001*[a]	*0.168*
Laryngitis, n (%) [number of episodes]	9 (6.6) [10]	11 (9.3) [13]	22 (18) [40]	*0.415*	*0.005*[a]	*0.05*
Tracheitis, n (%) [number of episodes]	36 (26.3) [44]	32 (27.1) [41]	49 (40.2) [76]	*0.880*	*0.018*	*0.033*

[a] The p-values remain significant after Bonferroni correction.

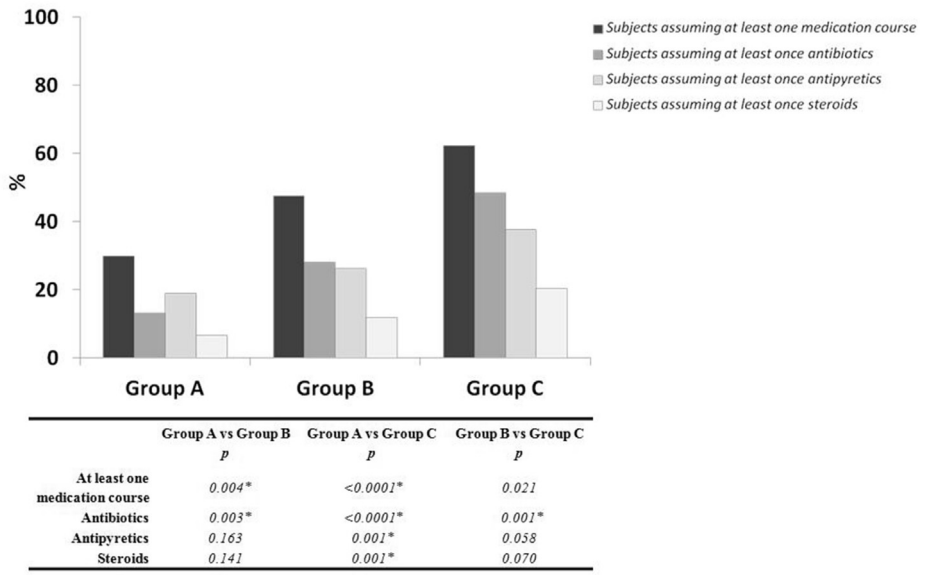
line of defense against pathogens in mucosal surface, but also they regulate gut microbiota composition driving the communication between gut commensal bacteria and the host immune system [18]. A positive effects on gut microbiota composition and function has been recently demonstrated in a human study, where a fermented milk product deriving from a mix of probiotic species was able to increase short chain fatty acids production and decrease the abundance of pathobionts [19]. The gut microbiota has profound influence at multiple levels, even on the development and maintenance of respiratory tract immunity. A recent Cochrane review concluded that probiotics are better than placebo in reducing the number of subjects experiencing episodes of acute URTI, antibiotic use and related school absence [20]. Similar results have been reported using fermented dairy products containing Lactobacilli probiotics in pediatric as well in elderly populations [21–23]. The exact mechanism of such effect is still largely undefined. It has been shown that the effects of fermented dairy products arise not only from whole microorganisms cell wall components (lipotheicoic acid), or from the cytoplasmic content (bacterial DNA), but also from metabolites such as peptides produced during fermentation [4]. Peptides derived from major cow's milk proteins during fermentation are potential modulators of immune system [24–28]. These data could be responsible, at least in part, for the slight but significant higher magnitude of several clinical effects observed in children receiving cow's milk fermented product. As potential influence of different matrix on probiotics metabolism could not be excluded, as suggested by others [26,29]. If confirmed in future studies, these findings will pave the way to new approaches in the use of probiotics added to different foods.

The importance of the immunoregulatory effect exerted by the fermented dietary products investigated in this study is supported by the significant inverse correlation observed evaluating the α-defensin, β-defensin, LL-37, and of secretory IgA levels with the number of CIDs.

The favorable low number of children to treat and the net reduction from 40 to 60% of total number of infections observed in this study suggest that the use of these dietary products could have relevant clinical, public health, and economic consequences. In addition, they do not contain living organisms and could therefore stored easily, and even though the risk of bacterial translocation appears limited to fragile young children population, this risk does not exist with these products. Lastly, the activity of fermented products do not depend on *in vivo* fermentation process, which can vary widely according to subjects and conditions, as may be the case for prebiotic-containing formulas.

5. Conclusion

Considering the results of this trial, it could be stated that the use of selected fermented dietary products can be recommended as a valid strategy in preventing CIDs in children attending



	Group A vs Group B *p*	Group A vs Group C *p*	Group B vs Group C *p*
At least one medication course	*0.004**	*<0.0001**	*0.021*
Antibiotics	*0.003**	*<0.0001**	*0.001**
Antipyretics	*0.163*	*0.001**	*0.058*
Steroids	*0.141*	*0.001**	*0.070*

**the p-values remain significant after Bonferroni correction*

Fig. 4. The rate of subjects requiring medication use (i.e., antibiotics, antipyretics, steroids) during the study period.

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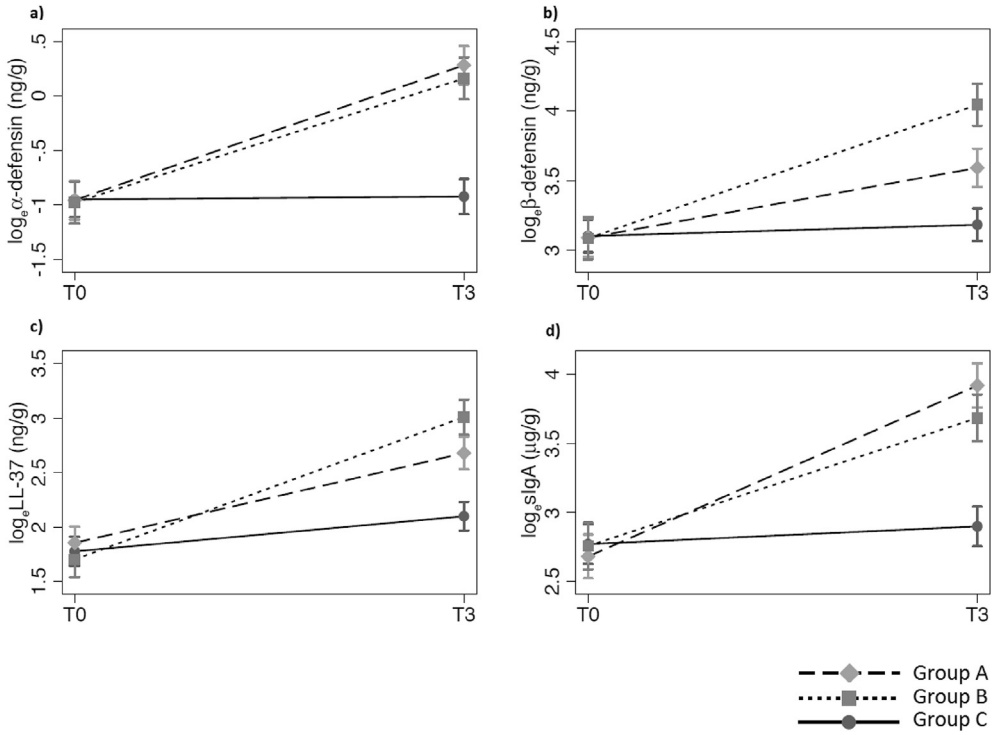

Fig. 5. Determination of innate and acquired immunity biomarkers at enrollment and after 3-month treatment in children evaluated in the three study groups. Panel a: alfa defensin; panel b: beta defensin; panel c: LL-37; panel d: sIgA. Values are means and 95% confidence intervals from random effect linear regression with correction for baseline.

educational program. Such benefits are even more interesting at a time when the number of children attending daycare centers has increased all over the world [30] and traditional medicine seems not to be completely able to provide adequate responses to the need for preventive strategy of disease using side effect-free treatments. But, it is important to recognize that this RCT studied specific fermented dietary products with well characterized probiotic strain, dose, and age group, and that our findings cannot be extrapolated for other strains.

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Conflict of interest

The authors have no conflict of interests that are directly relevant to the content of this paper, which remains their sole responsibility.

Clinical trial registration

Clinical Trials Protocol Registration System (ClinicalTrials.gov Identifier: NCT01909128).

Contributors' statement

R. Nocerino and R. Berni Canani conceptualized the study design, coordinated the research team and drafted the manuscript.

G. Terrin coordinated the research team.

V. Pezzella, L. Cosenza, G. Cecere, G. De Marco, M. Micillo, F. Albano, R. Nugnes, P. Ferri, G. Ciccarelli, G. Giaccio, R. Spadaro, Y. Maddalena and F. Berni Canani cared for the children.

L. Paparo and A. Amoroso performed laboratory analyses.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2015.12.004.

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