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ORIGINAL CONTRIBUTION

Supplementation of red palm olein-enriched biscuits improves levels of provitamin A carotenes, iron, and erythropoiesis in vitamin A-deficient primary schoolchildren: a double-blinded randomised controlled trial

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Abstract

Purpose Vitamin A deficiency (VAD) remains a significant contributor to childhood morbidity and mortality in developing countries; therefore, the implementation of sustainable and cost-effective approaches to control VAD is of utmost pertinence. This study aims to investigate the efficacy of red palm olein (RPO)-enriched biscuit supplementation in improving vitamin A, haematological, iron, and inflammatory status among vitamin A-deficient schoolchildren.

Methods We conducted a double-blinded, randomised controlled trial involving 651 rural primary schoolchildren (8–12 years) with VAD in Malaysia. The schoolchildren were randomised to receive either RPO-enriched biscuits (experimental group, $n = 334$) or palm olein-enriched biscuits (control group, $n = 317$) for 6-month duration.

Results Significant improvements in retinol and retinol-binding protein 4 levels were observed in both groups after supplementation ($P < 0.001$). The improvement in retinol levels were similar across groups among subjects with confirmed VAD ($P = 0.40$). Among those with marginal VAD, greater improvement in retinol levels was recorded in the control group ($P < 0.001$) but lacked clinical significance. The levels of α- and β-carotenes, haematological parameters (haemoglobin, packed cell volume, mean corpuscular volume and mean corpuscular haemoglobin) and iron enhanced more significantly in the experimental group ($P < 0.05$). The significant reduction in the prevalence of microcytic anaemia (− 21.8%) and high inflammation (− 8.1%) was only observed in the experimental group.

Conclusion The supplementation of RPO-enriched biscuits enhanced levels of provitamin A carotenes, iron, and erythropoiesis, and exhibited anti-inflammatory effects. Therefore, the incorporation of RPO into National Nutritional Intervention Programs may be a potential measure to improve the health status of vitamin A-deficient children, among various other interventions.

Clinical trial registration ClinicalTrials.gov (NCT03256123).

Keywords Red palm olein · Vitamin A deficiency · Carotene · Iron · Erythropoiesis · Inflammation

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Introduction

Vitamin A deficiency (VAD) is endemic in developing countries, affecting approximately 190 million preschool-age children in Africa and Southeast Asia [1]. It is a leading cause of preventable blindness, and affects normal haematopoiesis, iron metabolism and immune function [2]. The main underlying cause of VAD is chronically inadequate intake of vitamin A, which is normally found in animal source foods as preformed vitamin A (retinol and retinyl esters) and in plant source foods as provitamin A carotenes [1, 3]. There is a significant association between iron-deficiency anaemia (IDA) and VAD represented by low serum retinol level [4]. According to World Health Organization (WHO), iron-deficiency (ID) is the most common and widespread nutritional deficiency in the world, with 30% of the world's population are being anaemic due to ID, poor diet, or exposed to infectious diseases [5].

Despite great efforts to address VAD through oral administration of high-dose vitamin A capsules, there is still a need for additional approaches that are relatively more sustainable, long-term and cost-effective, such as dietary diversification, food fortification, multi-micronutrient powder, selective breeding and biofortification [6]. Since low-income populations tend to practice monotonous diets which are mainly energy dense with inadequate amount of vitamin A, food fortification can, therefore, be an attractive and potentially effective strategy to control VAD [7]. The supplementation using biscuits has been shown as one of the most effective and practical food vehicles for micronutrient-fortification feeding among primary schoolchildren due to its high acceptability as it is considered as a snack rather than a meal. Besides, it requires no preparation, is easily distributed and monitored, and has long shelf-life [8, 9].

High intake of vitamin A in the form of preformed vitamin A may impose a potential risk of hypervitaminosis A or vitamin A toxicity; however, this is unlikely to occur when provitamin A-rich foods are largely consumed [10]. Our group has previously reviewed that red palm olein (RPO) is the richest natural plant source of carotenoids (600–750 ppm), as well as an excellent source of other phytonutrients such as vitamin E, phytosterols, squalene and coenzyme Q10 [11]. It is produced from crude palm oil (CPO) via a milder refining process to retain about 80% of the carotenoids and vitamin E originally present in CPO [12]. Carotenoids can be cleaved to form retinal, which is further reduced to retinol (Vitamin A) or oxidised to retinoic acid (the most active form of vitamin A) in our body [13]. Therefore, RPO may serve a dual role in enhancing vitamin A status by providing both provitamin A carotenes and oil that facilitates the absorptions of both preformed vitamin A and provitamin A carotenes [14]. By replacing commercial shortening with RPO-based shortening in the production of biscuits, β-carotene and other phytonutrient compounds are naturally enhanced and synthetic fortification of β-carotene is hence devoided [15].

It was previously reported that RPO efficiently improved vitamin A status [16–20] and haemoglobin levels [21] among children with VAD. In this study, we aim to study the effect of RPO-enriched biscuit supplementation on vitamin A status, as well as haematological, iron, and inflammatory parameters of primary schoolchildren with VAD in rural areas of Malaysia.

Materials and methods

Study design

A double-blinded randomised controlled trial with a pre–post-test design was conducted among schoolchildren from ten national primary schools located in rural areas of five different states of Malaysia between April 2017 and June 2019. The criteria of school selection were described previously [22]. Clustered trials at the school level were carried out: in each state, a school received RPO-enriched biscuits (experimental school) while another school received palm olein (PO)-enriched biscuits (control school). Randomisation was carried out based on permutation using a computer-generated randomisation list. The students from different schools were matched for VAD status, age, sex, and soil-transmitted helminth (STH) infection status upon randomisation. The biscuits were packaged similarly across the groups in individual foil wrappers. The personnel who handled the biscuit distribution and processed biological samples including analyses were blinded to the supplementation groups, with the blinding procedure carried out by a third party (biscuit manufacturer), who is not involved in the study.

Given that most of the chosen areas were endemic for STH infections, all the subjects were dewormed through the administration of 3 doses (1 dose per day for 3 consecutive days) of 10 mL Vemizol suspension in which each 5 mL contains 200 mg of Albendazole, prior to the initiation of supplementation except for the schoolchildren from Sarawak who were given only one dose as there was no STH infection detected among them during the screening period. The follow-up period for supplementation lasted up to 6 months, with measurements of outcome variables taken at baseline and after 6 months of supplementation (endpoint). For the follow-up of the post-study, selected blood markers including retinol, retinol-binding protein 4 (RBP4), α-carotene, and β-carotene were analysed at 200 days after the completion

of the study, taken into consideration the long half-life of vitamin A (200–300 days) [23]. The study was conducted in accordance with the principles of the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (Identifier: NCT03256123) and ethical approval was obtained from the Medical Research Ethics Committee, Ministry of Health (MOH) Malaysia (NMRR No: NMRR-16-1905-32547). Written informed consents were obtained from all the literate parents before the study commencement. As for illiterate guardians, we obtained verbal consents with their thumbprint on the informed consent form. All the verbal consents were witnessed and formally recorded. An assent form was signed by the schoolchildren themselves.

Participants

Sample size was calculated based on an estimation of standard deviation of 0.3 µmol/L with a true difference of 0.15 µmol/L in serum retinol concentration after supplementation of RPO among schoolchildren one year duration in Burkina Faso [16]. Assuming a dropout rate of 40% in rural schools and VAD prevalence of 4.5% in Malaysia based on serum retinol concentrations of < 0.70 µmol/L [24], a sample size of at least 280 schoolchildren (140 schoolchildren per arm) with a power of 95% and a two-sided significance level of 0.05 is needed.

A total of 1164 schoolchildren were assessed for eligibility during the screening visit. Schoolchildren were included based on these criteria: aged 7–11 years (at screening), detected with confirmed VAD defined by retinol concentration < 0.70 µmol/L or marginal VAD defined by retinol concentration 0.70 to < 1.05 µmol/L [25], and not physically handicapped. Exclusion criteria included oedema including severe acute malnutrition or gastrointestinal disorders, allergic to wheat- and/or gluten-containing foods and studying in primary six. Schoolchildren aged 12 years or studying in primary six were not included as they were no longer be attending primary school when the intervention phase was initiated. After excluding 513 students (44.1%) who missed the screening examination or were not meeting inclusion criteria, 651 students were enrolled in the study. The schematic flow of the participants in this study is shown in Fig. 1.

Intervention method

Biscuits were prepared with RPO shortening (experimental group) or PO shortening (control group) and distributed to the subjects 4 days a week on schooling days. The manufacturing of the biscuits was conducted by a local biscuit company with close monitoring by the research team for quality control and assurance. The biscuits were formulated as alternating four flavours to provide a variety for a duration of 6 months. The production and delivery of the biscuits to the respective schools were conducted in two batches: before the initiation of the study and at 3-month supplementation, to ensure the stability of carotene content in the RPO-enriched biscuits, throughout the entire study duration.

RPO-enriched biscuits contain a substantially higher amount of total carotenes (389 µg/ml per 36 g serving) compared to PO-enriched biscuits (13 µg/ml per 36 g serving) (analysed using UV/Vis spectrophotometer [PerkinElmer Lambda 35, USA]). The biscuits of both groups were similar with respect to the vitamin E content (523 µg/ml per 36 g of RPO-enriched biscuits and 504 µg/ml per 36 g of PO-enriched biscuits) (analysed using high-performance liquid chromatography (HPLC) [Agilent 1100 Series, USA]), and macronutrient composition (23.8 g carbohydrate, 4.4 g protein and 11.8 g fat, contributing to 215 kcal per 36 g biscuit) (calculated using Nutritionist Pro™ software [AXXYA Systems LLC, Texas, USA]). In addition, the biscuits of both groups had correspondingly similar fatty acid composition (44% 16:0, 4% 18:0, 39% 18:1, and 10% 18:2) (analysed using gas chromatography [PerkinElmer, Inc., California, USA]). Schoolchildren in the experimental group received ~ 326.3 µg retinol activity equivalents (RAEs) for vitamin A/day on a weekly basis, fulfilling about 59.3% of the Recommended Dietary Allowance (RDA) for vitamin A in children aged 7–12 years [26]. The schoolchildren consumed all the biscuits given in front of the teachers during schooling days and in front of the guardians or field workers assigned during school holidays.

Study outcomes

Primary outcome was the change in plasma retinol concentration at 6 months from baseline. Secondary outcomes were the changes in RBP4, α-carotene, β-carotene, α-tocopherol (vitamin E), red blood cell (RBC) count, haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), serum ferritin, iron and high-sensitivity C-reactive protein (hs-CRP) levels at 6 months from baseline, as well as the changes in the selected blood markers, including retinol, RBP4, α-carotene and β-carotene at post-study from baseline and 6-month endpoint.

Data collection

Blood biochemical assessments

Approximately 6 mL of fasting blood was collected by trained nurses and medical assistants from MOH and covered with aluminium foil to prevent direct exposure to sunlight and kept in an icebox surrounded by ice. A complete blood count (CBC) was performed on a haematology

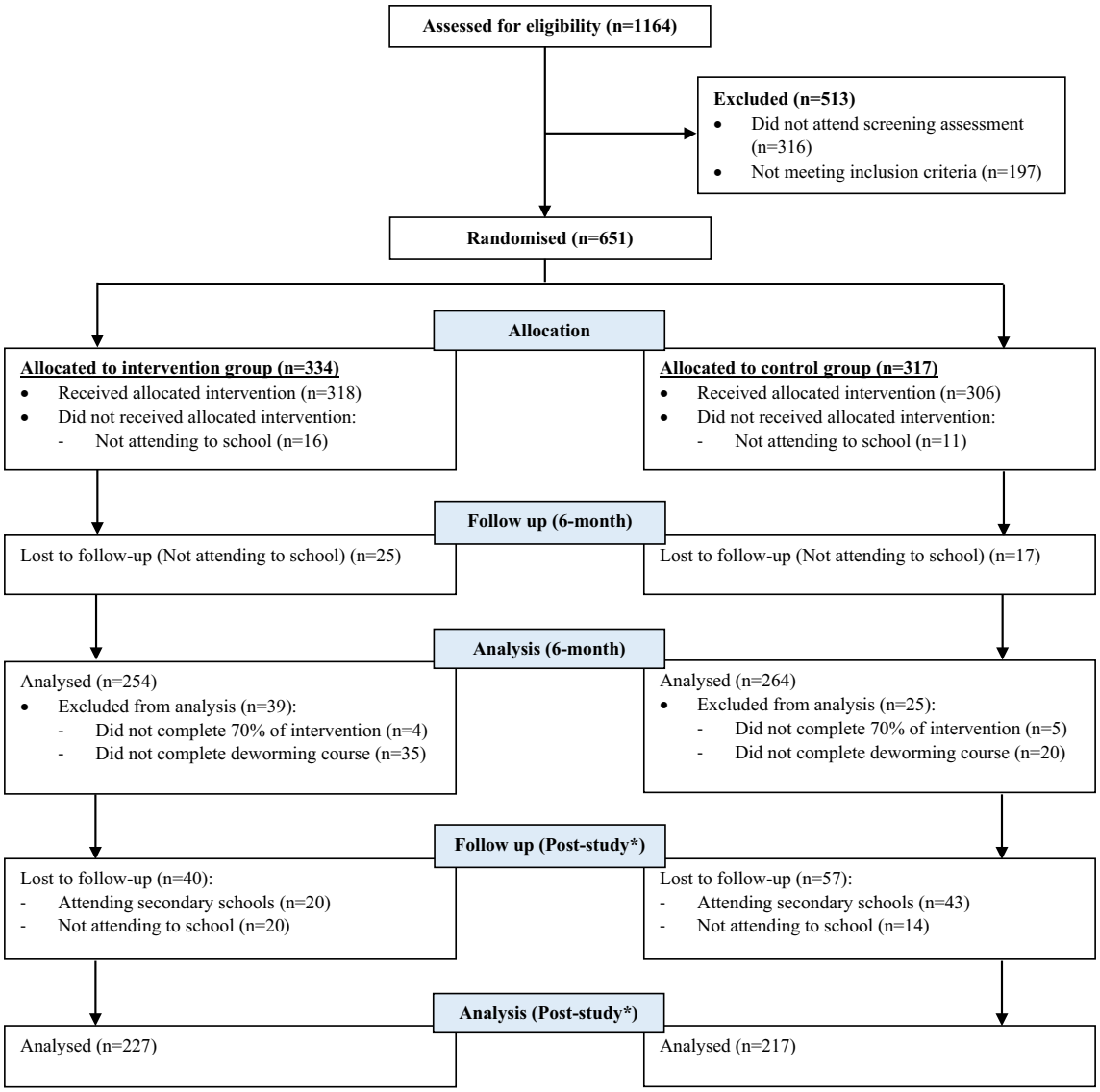

Fig. 1 Participants flow diagram. **Post-200 days after the completion of study*

autoanalyzer (Sysmex XN-2000, Kobe, Japan). The rest of the blood samples were centrifuged at 3000 rpm and 4 °C for 15 min to obtain serum and plasma, followed by storage at − 80 °C until further analysis.

The assessments of plasma retinol, α-carotene, β-carotene, and α-tocopherol levels were conducted using reverse-phase HPLC (Agilent 1260 Infinity, US) as described by Kand'ár et al. [27] with minor modifications following Loganathan et al. [28]. Briefly, plasma samples were extracted twice using hexane, by adding retinyl acetate and α-tocopheryl acetate as internal standards. The mean recovery of retinyl acetate and α-tocopheryl acetate were 93.34 ± 2.82% and 98.58 ± 3.66%, respectively. RBP4 was assessed by quantitative sandwich enzyme immunoassay (R&D Systems, USA). Low RBP4 was defined by concentrations at < 0.70 µmol/L [29].

Haematological determinations included RBC count, Hb, PCV, MCV and MCH. Anaemia and microcytic anaemia were identified when Hb concentration < 115 g/L and MCV < 80 FL, respectively [30, 31]. Serum ferritin, iron and hs-CRP were assessed using ADVIA 2400 Chemistry System and Centaur XP (Siemens). Serum ferritin < 15 µg/L was considered as ID, while IDA was identified when ID was diagnosed concurrently with anaemia [32, 33]. Children with serum iron concentrations < 11 µmol/L were considered as having low iron status, while those with hs-CRP concentration > 5.0 mg/L were considered as having a high inflammatory response [34]. The blood assessments including CBC,

serum iron and ferritin, as well as hs-CRP were performed at an accredited laboratory, Pathology & Clinical Laboratory (M) Sdn. Bhd., Malaysia.

Compliance records

The situation of biscuit distribution, effect of supplementation on the health and attendance of the students were recorded by teachers or trained field workers. Subjects who did not complete the full course of deworming, at least 70% of supplementation or both were excluded from the analysis.

Statistical analysis

Statistical analysis was carried out based on the principle of per-protocol analysis. Continuous variables were presented as mean and standard deviation (mean ± SD) or 95% confidence interval (95% CI). The effects of the supplementation on blood biochemical parameters were tested on the response variable in a linear mixed-effects model (LMM) fitted by restricted maximum likelihood estimation method (REML), using the *lme* function from the *nlme* package. Supplementation group, timepoint, and their interaction term (group x timepoint), as well as state, school type, age and sex were included into the model as fixed effects, with subject added as a random effect. The statistical significance of the group x timepoint interaction term was estimated by Type III analysis of variance (ANOVA) via the *anova.lme* function from the *nlme* package. Post hoc pairwise assessment of LMM was performed via the *emmeans* function from the *emmeans* package with *P* value adjustment using Bonferroni corrections to evaluate the changes in outcomes across timepoints within the same group. Given that only 2.5% of the variance in retinol (based on intraclass correlation coefficient [ICC]), which is the primary outcome of this study, was explained by school, it was not adjusted for clustering in our analysis. Besides, the inclusion of school as fixed effect did not further improve the LMM analysis on retinol concentration; therefore, it was not included in the LMM model for covariate adjustment. When baseline difference between groups was observed, an analysis of covariance (ANCOVA) was conducted with the endpoint value as the response variable, and baseline value, group, state, school type, age, and sex as predictor variables. The distribution normality of the residuals from both the LMM and ANCOVA were checked via the *plot_model* function from the *sjPlot* package. Subsequent subgroup analyses were used to measure the effect of supplementation on VAD-related indicators: retinol and RBP4 markers by baseline VAD status. The categorical data were presented as numbers and percentages. To determine significant changes in the proportion of subjects with confirmed VAD, low RBP4, anaemia, ID, IDA, microcytic anaemia, low iron and high inflammation between the baseline and endpoint of each group, the McNemar's test was applied. A P value < 0.05 was considered statistically significant. RStudio (R version 4.1.2, Vienna, Austria) was used for all analyses.

Results

Baseline characteristics

Of the 651 schoolchildren (310 boys and 341 girls) recruited for the study, 607 completed the supplementation (Fig. 1). No subjects reported adverse health conditions due to supplementation nor withdrew from the trial due to adverse events. Baseline characteristics of the subjects enrolled in each group are presented in Table 1. Majority of the subjects (72.8%) recruited were Orang Asli schoolchildren. Orang Asli, who transliterates as original people in the Malay language, is the indigenous minority population of Peninsular Malaysia as described in our previous published data [22]. The mean age of the overall recruited subjects was 10.0 ± 1.4 years. The distributions of age and sex were similar across groups. Overall, 173 subjects (26.6%) were diagnosed with confirmed VAD, while 478 (73.4%) were found to have marginal VAD. The prevalence of VAD (experimental group: 27.8% confirmed VAD and 72.2% marginal VAD; control group: 25.2% confirmed VAD and 74.8% marginal VAD) at baseline did not differ between the experimental and control groups.

Changes in vitamin A deficiency indicators and antioxidants

Based on Tables 2 and 3, significant increase in retinol concentrations [experimental group: from 0.80 µmol/L, 95% CI (0.78, 0.82) to 0.84 µmol/L, 95% CI (0.82, 0.87), $P = 0.003$; control group: from 0.81 µmol/L, 95% CI (0.80, 0.83) to 0.92 µmol/L, 95% CI (0.90, 0.94), $P < 0.001$], and reduction in the proportion of subjects with confirmed VAD (retinol < 0.70 µmol/L) (experimental group: − 9.4%, $P = 0.007$; control group: − 12.5%, $P < 0.001$) were observed in both groups after 6-month supplementation program. A significant interaction between group x timepoint was noted for retinol concentration, indicating that the changes in retinol concentration across timepoints were significantly different between groups ($P < 0.001$) (Table 2).

Further analysis was conducted based on the baseline VAD status (marginal and confirmed VAD). Significant improvements in retinol concentrations were found in schoolchildren with confirmed VAD from both the experimental [from 0.58 µmol/L, 95% CI (0.56, 0.60) to 0.76 µmol/L, 95% CI (0.72, 0.80); $P < 0.001$] and control groups [from 0.61 µmol/L, 95% CI (0.59, 0.63) to

Table 1 Baseline characteristics of schoolchildren participating in the 6-month RPO-enriched biscuit supplementation programme ($N=651$)

	Experimental group ($n=334$)	Control group ($n=317$)	Total ($N=651$)
Socio-demographic characteristics			
Age (years)	10.0 ± 1.4	10.0 ± 1.4	10.0 ± 1.4
Sex			
Male	154 (46.1)	156 (49.2)	310 (47.6)
Female	180 (53.9)	161 (50.8)	341 (52.4)
School type			
Orang Asli[a] schools	275 (82.3)	199 (62.8)	474 (72.8)
Non-Orang Asli schools	59 (17.7)	118 (37.2)	177 (27.2)
State			
Pahang	163 (48.8)	110 (34.7)	273 (41.9)
Perak	68 (20.4)	89 (28.1)	157 (24.1)
Johor	44 (13.2)	34 (10.7)	78 (12.0)
Sabah	29 (8.7)	35 (11.0)	64 (9.8)
Sarawak	30 (9.0)	49 (15.5)	79 (12.1)
Monthly household income			
< RM500	166 (64.3)	160 (63.5)	326 (63.9)
≥ RM500	92 (35.7)	92 (36.5)	184 (36.1)
Maternal education			
Post-secondary education	5 (1.9)	7 (2.8)	12 (2.4)
Secondary education	77 (29.7)	88 (35.8)	165 (32.7)
Primary education	121 (46.7)	101 (41.1)	222 (44.0)
Not received education	56 (21.6)	50 (20.3)	106 (21.0)
Paternal work status			
Working	225 (91.5)	213 (89.5)	438 (90.5)
Non-working	21 (8.5)	25 (10.5)	46 (9.5)
Maternal work status			
Working	71 (28.1)	83 (33.7)	154 (30.9)
Non-working	182 (71.9)	163 (66.3)	345 (69.1)
Biochemical indicators			
Retinol (μmol/L)	0.80 ± 0.16	0.81 ± 0.15	0.80 ± 0.16
RBP4 (μmol/L)	0.68 ± 0.18	0.73 ± 0.16	0.71 ± 0.17
α-Carotene (μg/mL)	0.06 ± 0.06	0.07 ± 0.05	0.07 ± 0.05
β-Carotene (μg/mL)	0.30 ± 0.18	0.36 ± 0.21	0.32 ± 0.20
α-Tocopherol (μg/mL)	7.73 ± 1.72	8.04 ± 1.60	7.88 ± 1.67
RBC ($\times 10^{12}$/L)	5.1 ± 0.5	5.2 ± 0.5	5.1 ± 0.5
Hb (g/L)	122.3 ± 12.2	124.3 ± 11.4	123.3 ± 11.8
PCV (%)	37.3 ± 3.4	38.6 ± 4.0	37.9 ± 3.7
MCV (FL)	73.9 ± 8.3	74.9 ± 8.9	74.4 ± 8.7
MCH (PG)	24.3 ± 3.0	24.4 ± 2.9	24.3 ± 2.9
Iron (μmol/L)	11.9 ± 6.1	11.4 ± 5.1	11.7 ± 5.6
Ferritin (μg/L)	47.4 ± 37.9	41.9 ± 28.1	44.7 ± 33.5
hs-CRP (mg/L)	2.8 ± 9.6	2.1 ± 7.1	2.5 ± 8.5
VAD status			
Confirmed (Retinol < 0.70 μmol/L)	93 (27.8)	80 (25.2)	173 (26.6)
Marginal (Retinol 0.70—< 1.05 μmol/L)	241 (72.2)	237 (74.8)	478 (73.4)
Low RBP4 (RBP4 < 0.70 μmol/L)	195 (58.7)	138 (43.7)	333 (51.4)
Anaemia (Hb < 115 g/L)	63 (19.1)	58 (18.6)	121 (18.9)
Iron-deficiency (Ferritin < 15 μg/L)	52 (16.0)	52 (16.6)	104 (16.3)
Iron-deficiency anaemia (Hb < 115 g/L and ferritin < 15 μg/L)	33 (10.3)	19 (6.1)	52 (8.2)

Table 1 (continued)

	Experimental group ($n=334$)	Control group ($n=317$)	Total ($N=651$)
Microcytic anaemia (MCV < 80 FL)	236 (71.7)	207 (66.3)	443 (69.1)
Low iron (Iron < 11 µmol/L)	160 (49.2)	161 (51.4)	321 (50.3)
High inflammation (hs-CRP > 5.0 mg/L)	48 (14.8)	24 (7.7)	72 (11.3)

Values are given as mean ± standard deviation or *n* (%). All data were collected at enrolment

VAD Vitamin A deficiency, *RBP4* retinol-binding protein 4, *RBC* red blood cell, *Hb* haemoglobin, *PCV* packed cell volume, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *hs-CRP* high-sensitivity C-reactive protein, *STH* soil-transmitted helminths

[a]Indigenous minority peoples of Peninsular Malaysia

0.82 µmol/L, 95% CI (0.78, 0.86); $P<0.001$], but no significant interaction between the group x timepoint was found ($P=0.40$). Among schoolchildren with marginal VAD, no significant change in retinol concentration was observed in the experimental group ($P>0.99$), while the control group recorded a significant improvement in retinol concentration after the supplementation ($P<0.001$). However, this mean change in retinol concentration for control group [0.08 µmol/L, 95% CI (0.05, 0.10)] was more than 2.0-fold lower than the mean changes observed among the schoolchildren with confirmed VAD, regardless of the group assigned [experimental group: 0.18 µmol/L, 95% CI (0.14, 0.23); control group: 0.21 µmol/L, 95% CI (0.17, 0.25)].

Similarly, we observed significant improvements in RBP4 concentrations [experimental group: from 0.68 µmol/L, 95% CI (0.66, 0.70) to 0.75 µmol/L, 95% CI (0.73, 0.77), $P<0.001$; control group: from 0.73 µmol/L, 95% CI (0.72, 0.75) to 0.79 µmol/L, 95% CI (0.77, 0.81), $P<0.001$] and a significantly lower proportion of subjects with low RBP4 status (RBP4 < 0.70 µmol/L) in both groups (experimental group: − 19.8%, $P<0.001$; control group: − 11.4%; $P=0.002$) after 6-month supplementation, as compared to their respective baseline levels (Tables 2 and 3). However, the group x timepoint interaction for RBP4 concentration was not statistically significant ($P=0.32$) (Table 2). Based on subgroup analysis by baseline VAD status, significant improvements in RBP4 concentrations among confirmed VAD subjects were observed in both the experimental [from 0.54 µmol/L, 95% CI (0.51, 0.57) to 0.68 µmol/L, 95% CI (0.65, 0.72), $P<0.001$] and control group [from 0.65 µmol/L, 95% CI (0.62, 0.69) to 0.71 µmol/L, 95% CI (0.67, 0.76), $P=0.01$]. However, there was no significant difference in RBP4 concentrations between the experimental and control groups at the endpoint of 6-month supplementation after baseline adjustment ($P=0.17$).

Besides, significant improvements in α- and β-carotene levels were observed only in the experimental group after a 6-month supplementation, with the mean change of α-carotene [0.32 µg/mL, 95% CI (0.28, 0.35)] and β-carotene [0.65 µg/mL, 95% CI (0.61, 0.70)] being 21.7- and 16-fold higher than the control group [α-carotene: 0.03 µg/mL, 95% CI (0.02, 0.04); β-carotene: 0.02 µg/mL, 95% CI (− 0.01, 0.04)], respectively ($P<0.001$). After adjusting for baseline, the β-carotene level in the experimental group was significantly higher than that of the control group at the 6 months of supplementation ($P<0.001$). No statistical difference in the group x timepoint interaction was observed for α-tocopherol ($P=0.15$), indicating both groups demonstrated similar improvement in α-tocopherol after the 6-month supplementation.

Changes in haematological, iron and inflammatory parameters

After 6-month supplementation of RPO-enriched biscuits, the experimental group recorded significant interaction of group x timepoint for various haematological parameters, including Hb ($P=0.01$), MCV ($P<0.001$) and MCH ($P=0.003$), except RBC counts ($P>0.99$) (Table 2). In addition to that, PCV level was significantly higher in the experimental group than the control group at 6 months of supplementation after adjusting with baseline values ($P<0.001$). We also observed the mean change in serum iron was four-fold higher in the experimental group [3.2 µmol/L, 95% CI (2.1, 4.2)] than in the control group [0.8 µmol/L, 95% CI (0.1, 1.5)], with a significant group x timepoint interaction ($P<0.001$). Inversely, the experimental group [− 6.7 µg/L, 95% CI (− 13.9, 0.5)] exhibited a greater drop in ferritin level compared to the control group [− 1.8 µg/L, 95% CI (− 5.5, 1.9)], but no significant interaction of group x timepoint was observed ($P=0.23$).

Based on Table 3, in both groups, significant reductions in the prevalence of anaemia (haemoglobin < 115 g/L) were found in both the experimental (− 6.1%, $P=0.012$) and control (− 8.2%, $P=0.001$) groups after 6-month supplementation. However, no significant changes in the prevalence of IDA (haemoglobin < 115 g/L and ferritin < 15 µg/L) were observed in both the experimental ($P=0.08$) and control ($P=0.21$) groups after the supplementation. In addition, there was a significant reduction in the prevalence of microcytic anaemia (MCV < 80 FL) among the subjects from the experimental group (− 21.8%, $P<0.001$), but no significant

Table 2 Biomarker measurement of schoolchildren participating in the 6-month RPO-enriched biscuit supplementation programme ($N=518$)

Variables	Group	n	Baseline	6-month (end-point)	Mean change	*P* value[a] (group x time-point)	*P* value[b] (within group)	*P* value[c] (baseline-adjusted)
VAD indicators								
Retinol (μmol/L)	Experimental	254	0.80 (0.78, 0.82)	0.84 (0.82, 0.87)	0.04 (0.02, 0.07)	< 0.001	0.003	–
	Control	264	0.81 (0.80, 0.83)	0.92 (0.90, 0.94)	0.11 (0.08, 0.13)		< 0.001	
Retinol by baseline VAD status (μmol/L)								
Confirmed VAD*	Experimental	72	0.58 (0.56, 0.60)	0.76 (0.72, 0.80)	0.18 (0.14, 0.23)	0.40	< 0.001	–
	Control	63	0.61 (0.59, 0.63)	0.82 (0.78, 0.86)	0.21 (0.17, 0.25)		< 0.001	
Marginal VAD*	Experimental	182	0.89 (0.88, 0.90)	0.88 (0.85, 0.90)	− 0.01 (− 0.04, 0.01)	< 0.001	> 0.99	–
	Control	201	0.88 (0.86, 0.89)	0.95 (0.92, 0.98)	0.08 (0.05, 0.10)		< 0.001	
RBP4 (μmol/L)	Experimental	243	0.68 (0.66, 0.70)	0.75 (0.73, 0.77)	0.07 (0.05, 0.09)	0.32	< 0.001	-
	Control	261	0.73 (0.72, 0.75)	0.79 (0.77, 0.81)	0.05 (0.03, 0.08)		< 0.001	
RBP4 by baseline VAD status (μmol/L)								
Confirmed VAD*	Experimental	69	0.54 (0.51, 0.57)	0.68 (0.65, 0.72)	0.14 (0.11, 0.18)	0.002	< 0.001	0.17
	Control	62	0.65 (0.62, 0.69)	0.71 (0.67, 0.76)	0.06 (0.02, 0.10)		0.01	
Marginal VAD*	Experimental	174	0.74 (0.71, 0.76)	0.78 (0.75, 0.81)	0.04 (0.01, 0.07)	0.55	0.02	–
	Control	199	0.76 (0.74, 0.78)	0.81 (0.79, 0.84)	0.05 (0.03, 0.08)		< 0.001	
Other antioxidant profile								
α-Carotene (μg/mL)	Experimental	254	0.07 (0.06, 0.07)	0.72 (0.67, 0.77)	0.65 (0.61, 0.70)	< 0.001	< 0.001	–
	Control	264	0.07 (0.06, 0.08)	0.10 (0.09, 0.11)	0.03 (0.02, 0.04)		0.32	
β-Carotene (μg/mL)	Experimental	254	0.30 (0.28, 0.32)	0.61 (0.58, 0.65)	0.32 (0.28, 0.35)	< 0.001	< 0.001	< 0.001
	Control	264	0.35 (0.32, 0.37)	0.37 (0.34, 0.39)	0.02 (− 0.01, 0.04)		> 0.99	
α-Tocopherol (μg/mL)	Experimental	254	7.76 (7.54, 7.98)	8.86 (8.66, 9.05)	1.10 (0.90, 1.29)	0.15	< 0.001	–
	Control	264	7.97 (7.77, 8.17)	8.87 (8.66, 9.07)	0.90 (0.71, 1.09)		< 0.001	
Haematological, iron and inflammatory profile								
RBC count ($\times 10^{12}$/L)	Experimental	247	5.1 (5.1, 5.2)	5.2 (5.2, 5.3)	0.1 (0.1, 0.1)	> 0.99	< 0.001	–
	Control	257	5.2 (5.1, 5.2)	5.3 (5.2, 5.3)	0.1 (0.1, 0.1)		< 0.001	
Hb (g/L)	Experimental	247	122.9 (121.5, 124.3)	128.3 (126.9, 129.7)	5.4 (4.4, 6.5)	0.01	< 0.001	–
	Control	257	124.5 (123.1, 125.9)	127.9 (126.5, 129.4)	3.5 (2.4, 4.6)		< 0.001	
PCV (%)	Experimental	247	37.4 (37.0, 37.8)	40.3 (39.8, 40.7)	2.8 (2.5, 3.1)	< 0.001	< 0.001	< 0.001
	Control	257	38.5 (38.0, 39.0)	39.2 (38.8, 39.6)	0.7 (0.2, 1.1)		0.004	
MCV (FL)	Experimental	247	74.0 (72.9, 75.0)	77.7 (76.6, 78.7)	3.7 (3.2, 4.2)	< 0.001	< 0.001	–
	Control	257	74.5 (73.4, 75.6)	75.0 (74.0, 75.9)	0.4 (− 0.2, 1.0)		0.74	
MCH (PG)	Experimental	247	24.3 (23.9, 24.7)	24.9 (24.5, 25.2)	0.6 (0.4, 0.7)	0.003	< 0.001	–
	Control	257	24.4 (24.0, 24.7)	24.5 (24.2, 24.9)	0.2 (0.0, 0.4)		0.12	
Iron (μmol/L)	Experimental	249	12.0 (11.2, 12.7)	15.1 (14.2, 16.0)	3.2 (2.1, 4.2)	< 0.001	< 0.001	–
	Control	254	11.4 (10.8, 12.1)	12.2 (11.6, 12.9)	0.8 (0.1, 1.5)		0.48	
Ferritin (μg/L)	Experimental	248	49.1 (44.2, 54.0)	42.4 (35.8, 49.0)	− 6.7 (− 13.9, 0.5)	0.23	0.13	–
	Control	254	43.9 (40.4, 47.5)	42.2 (38.7, 45.7)	− 1.8 (− 5.5, 1.9)		> 0.99	
hs-CRP (mg/L)	Experimental	249	2.7 (2.0, 3.4)	2.3 (0.7, 3.9)	− 0.4 (− 2.1, 1.4)	0.80	> 0.99	–

Table 2 (continued)

Variables	Group	n	Baseline	6-month (endpoint)	Mean change	*P* value[a] (group x timepoint)	*P* value[b] (within group)	*P* value[c] (baseline-adjusted)
	Control	254	2.1 (1.2, 3.1)	1.5 (1.0, 2.0)	− 0.6 (− 1.7, 0.5)		> 0.99	

Values are given as unadjusted mean (95% confidence interval)

VAD vitamin A deficiency; RBP4, retinol-binding protein 4, *RBC* red blood cell, *Hb* haemoglobin, *PCV* packed cell volume, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *hs-CRP* high-sensitivity C-reactive protein

[a]Estimated by type III analysis of variance (ANOVA) on the linear mixed-effects model (LMM) including group, timepoint, group x timepoint interaction, state, school type, age and sex as fixed effects, with subject as a random effect

[b]Post hoc pairwise assessment of LMM (group, timepoint, group x timepoint interaction, state, school type, age and sex as fixed effects, and subject as a random effect) performed by contrast analysis with *P* value adjustment using Bonferroni corrections to evaluate the changes of outcomes from baseline to endpoint within the same group

[c]Estimated using analysis of covariance (ANCOVA) with baseline value, group, state, school type, age and sex as predictor values

[*]VAD status by baseline retinol concentration (confirmed VAD: < 0.70 µmol/L; marginal VAD: 0.70—< 1.05 µmol/L)

Table 3 Changes in the proportion of schoolchildren with micronutrient deficiencies, anaemia, high inflammation and STH infections ($N = 518$)

	Experimental group ($n = 254$)				Control group ($n = 264$)			
	n	Baseline	6-month (endpoint)	*P* value[a]	*n*	Baseline	6 months (endpoint)	*P* value[a]
VAD indicators								
Confirmed VAD (Retinol < 0.7 µmol/L)	254	72 (28.3)	48 (18.9)	0.007	264	63 (23.9)	30 (11.4)	< 0.001
Low RBP4 (RBP4 < 0.7 µmol/L)	243	146 (60.1)	98 (40.3)	< 0.001	261	116 (44.4)	86 (33.0)	0.002
Haematological, iron and inflammatory profile								
Anaemia (Hb < 115 g/L)	247	45 (18.2)	30 (12.1)	0.012	257	49 (19.1)	28 (10.9)	0.001
Iron-deficiency (Ferritin < 15 µg/L)	248	36 (14.5)	51 (20.6)	0.007	254	43 (16.9)	28 (11.0)	0.02
Iron-deficiency anaemia (Hb < 115 g/L and ferritin < 15 µg/L)	243	25 (10.3)	17 (7.0)	0.08	251	14 (5.6)	8 (3.2)	0.21
Microcytic anaemia (MCV < 80 FL)	247	174 (70.4)	120 (48.6)	< 0.001	257	175 (68.1)	170 (66.1)	0.53
Low iron (Iron < 11 µmol/L)	249	124 (49.8)	75 (30.1)	< 0.001	254	128 (50.4)	107 (42.1)	0.03
High inflammation (hs-CRP > 5.0 mg/L)	249	38 (15.3)	18 (7.2)	0.003	254	19 (7.5)	22 (8.7)	0.74

Values are given as *n* (%)

*VAD*Vitamin A deficiency, *RBP4* retinol-binding protein 4, *Hb* haemoglobin; MCV, mean corpuscular volume, *hs-CRP* high-sensitivity C-reactive protein

[a]McNemar's test was performed to evaluate the changes of proportions for outcomes from baseline to endpoint within the same group

change was recorded in the control group (− 2.0%, $P = 0.53$). We observed a significant drop in the proportion of subjects with low iron status (iron < 11 µmol/L) in the experimental group (− 19.7%, $P = 0.03$), which was 2.3-fold higher than the reduction recorded in the control group (− 8.3%, $P < 0.05$). However, a contradictory pattern was observed for ID (ferritin < 15 µg/L), where ID was significantly increased in the experimental group (+ 6.1%, $P = 0.007$), while significantly decreased in the control group (− 5.9%, $P = 0.02$).

No significant group x timepoint interaction was observed for hs-CRP level ($P = 0.80$), indicating that the changes in hs-CRP levels across timepoints were similar among both the experimental and control groups (Table 2). However, a significant reduction in the proportion of subjects with high inflammation (hs-CRP > 5.0 mg/L) was observed in the experimental group (− 8.1%, $P = 0.003$), while no significant difference was recorded in the control group (+ 1.2%, $P = 0.74$) (Table 3).

Changes in VAD-related indicators and carotenes at post-study

Significant increments in retinol concentrations 200 days after the completion of study were observed in both the experimental [+ 0.10 µmol/L, 95% CI (0.07, 0.12); $P < 0.001$] and control group [+ 0.08 µmol/L, 95% CI (0.05, 0.10); $P < 0.001$] as compared to their respective baseline levels (Fig. 2). However, there was no significant interaction of group x timepoint for retinol concentrations ($P = 0.07$), indicating that the changes in retinol concentrations across

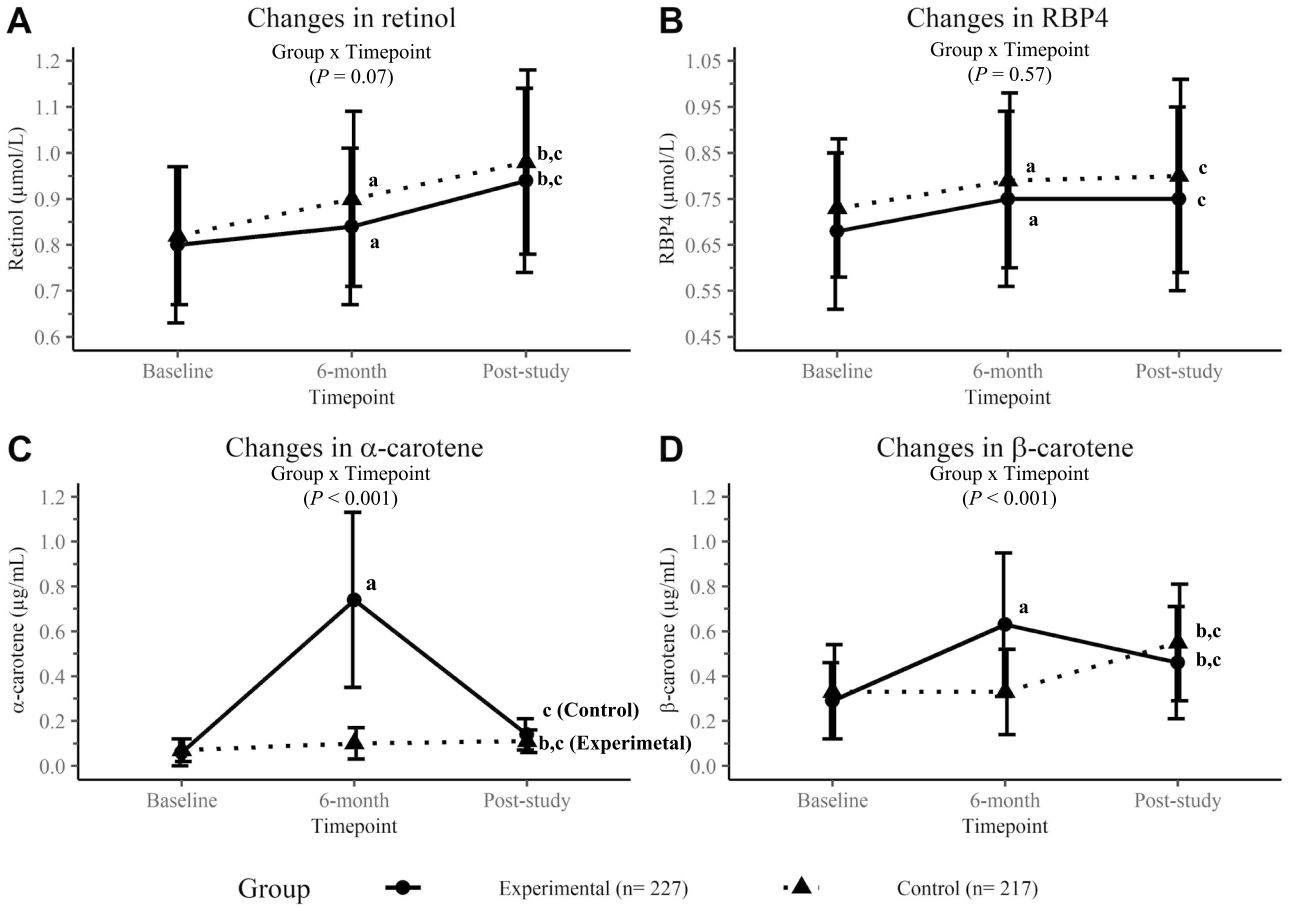

Fig. 2 Changes in VAD-related indicators and carotenes from baseline to post-study. **A** Changes in retinol concentration. **B** Changes in RBP4 concentration. **C** Changes in α-carotene concentration. **D** Changes in β-carotene concentration. The type of shape and lines indicate different group: filled circle with solid lines refer to experimental group, while filled triangle with dotted lines refer control group. The significance of group x timepoint interaction was evaluated by type III analysis of variance (ANOVA) on the linear mixed-effects model (LMM) including group, timepoint, group x timepoint interaction, state, school type, age and sex as fixed effects, with subject as a random effect. Different letters indicate statistically significant differences between timepoints within the same group: a = baseline versus 6-month, b = 6-month versus post-study, and c = baseline versus post-study, based on the post hoc pairwise assessment of LMM after Bonferroni corrections. *VAD* vitamin A deficiency, *RBP4* retinol-binding protein 4

three timepoints (baseline, 6 months, and post-study) were similar among the experimental and control groups. Similarly, the group x timepoint interaction for RBP4 concentration was not statistically significant ($P = 0.57$).

On the other hand, significant interactions between group and timepoint for both α- and β-carotene levels were noted ($P < 0.001$). Among subjects in the experimental group, there were significant reductions in both α-carotene [-0.60 µmol/L, 95% CI (− 0.65, − 0.55), $P < 0.001$] and β-carotene [− 0.17 µmol/L, 95% CI (− 0.21, − 0.13), $P < 0.001$] levels from the endpoint of 6-month supplementation to post-study, despite significantly higher levels of α-carotene (by 2.3-fold) and β-carotene (by 1.6-fold) were observed at post-study as compared to their respective baseline. Interestingly, a significant increase in β-carotene [+ 0.22 µmol/L, 95% CI (0.19, 0.25)] was noted in the control group from the endpoint of 6-month supplementation to the post-study phase ($P < 0.001$).

Discussion

The present study showed marked improvements in retinol and RBP4 status in both the experimental and control groups after 6 months of supplementation, notwithstanding the fact that the control group received PO-enriched biscuits containing negligible quantities of carotenes. These findings could be explained by the reduction in intestinal parasitic loads after deworming treatment in both groups (results have been published in [35]), which may have resulted in better

absorption of provitamin A carotenes or/and preformed vitamin A from their daily diet [36, 37]. The improved vitamin A status after the supplementation may also be attributable to the increased dietary fat provided in the biscuits of both treatment groups by enhancing bioavailability of carotenes in the human body [38]. Besides, with the knowledge that their children were vitamin A-deficient during the recruitment into this study, the parents of the schoolchildren may have included more vitamin A-rich foods in their diets at home, which could be potentially account for the observed improvement in the vitamin A status among the control subjects.

It was previously reported that the bioconversion of carotenes to vitamin A is inversely related to the baseline vitamin A status [36, 39]. Hence, the changes in retinol and RBP4 concentrations were further examined based on the baseline vitamin A status (based on retinol concentration). Among the schoolchildren with confirmed VAD at baseline, retinol levels elevated significantly in both groups after supplementation, with similar changes observed across timepoints among the groups. On the other hand, among schoolchildren with marginal VAD at baseline, the experimental group did not exhibit any significant change in the retinol concentration. Although there was a statistically significant improvement (by 9.1%) recorded in the control group, it is worth noting that the improvement lacked clinical significance if compared with the schoolchildren with confirmed VAD in the current study (by 31.0% in the experimental group and by 34.4% in the control group), as well as in previously reported RPO supplementation programmes, which ranged from 15.4 to 52.1% of increments [16, 40, 41]. Consistent findings were observed in the RBP4 levels. The lack of improvement in vitamin A status among schoolchildren with marginal VAD may be attributable to the achievement of homeostatic range of retinol in the bloodstream, whereby the circulating retinol remains relatively constant until the liver reserve is in excess or depletion [42]. It was previously reported that retinol increased and maintained in a narrow range of 0.70–1.05 µmol/L among undernourished children on moderate carotene diet [43]. Previous studies also recorded similar observations among schoolchildren with marginal VAD after vitamin A and carotene intervention [39, 44–46].

The fact that the compliance of schoolchildren to study protocol was reflected in the increased plasma α- and β-carotene levels following the 6-month supplementation of RPO-enriched biscuits, affirming the bioavailability of α- and β-carotenes found in RPO, which are consistent with the findings from our previous study in adults [28] and other RPO supplementation programmes [14, 47]. The marked increase in the circulating provitamin A carotenes suggests their bioconversion potential to retinol, especially under nutritional stress [39]. At post-study phase, the increase in retinol concentration from the endpoint of 6-month supplementation was statistically significant in both groups but lack clinical significance. This finding could also be attributed to the achievement of homeostatic range of retinol (0.70–1.05 µmol/L) after the supplementation. Given the relatively short half-lives of α- and β-carotenes at approximately 45 and 37 days, respectively, significant decline in both α- and β-carotene levels during the 200-day period of post-supplementation of RPO-enriched biscuits was anticipated [48]. Nevertheless, intriguingly, it was observed that β-carotene level in the control group enhanced significantly at post-study as compared to the endpoint of the 6-month supplementation, which suggests that a higher intake of carotenoid-rich foods was plausible in the control group after the completion of the study.

A significant improvement in serum iron status was found among the subjects supplemented with RPO-enriched biscuits. This could be explained by the high β-carotene content present in the RPO-enriched biscuits, which may have led to an improved absorption of iron from their daily diet. This finding is in agreement with the previous in vitro and human studies which reported an enhancement of iron absorption following the addition of carotenoids [49, 50]. Ferritin is known as a protein responsible for the storage of iron, which plays an important role in cellular iron homeostasis [51]. The improved iron mobilisation from hepatic stores into blood circulation was further affirmed by a significant reduction in ferritin level and a higher occurrence of ID after the supplementation of RPO-enriched biscuits. Similarly, several studies have also demonstrated a decline in ferritin status after receiving vitamin A supplementation [52, 53].

In addition, there were marked improvements in several haematological parameters, including Hb, PCV, MCV, and MCH, as well as a significant reduction in the occurrence of microcytic anaemia among the subjects supplemented with RPO-enriched biscuits, as compared to the control group. These outcomes indicate that the supplementation of RPO-enriched biscuits has potential beneficial effects on erythropoiesis. Our findings support the suggestion by Maramag et al. [54], where carotenoids may exert compartmentalised effects on iron metabolism by promoting the incorporation of iron into Hb. Similar observations were made in previous carotene supplementation programmes [46, 55]. In the present study, the significant drop in the proportion of subjects exhibiting high inflammation status (hs-CRP < 0.5 mg/L) was observed among the schoolchildren supplemented with RPO-enriched biscuits. This outcome suggests the potential anti-inflammatory effects of RPO, which may be attributed to its high phytonutrients content [11]. However, due to the small sample number of schoolchildren with high inflammation at baseline (experimental group: 15.3; control group: 7.5%), further studies with larger sample size are necessary to confirm this finding.

The study was well complied by the schoolchildren as indicated by the increased circulating plasma carotene levels. The strictly conducted double-blinded procedure has given extra strength to the study outcome credibility. The measures of both retinol and RBP4 levels as indicators of vitamin A status has further provided mechanistic insights. Unfortunately, the utilisation of retinol as the primary outcome was found to be lacked sensitivity and responsiveness to detect changes in vitamin A status as > 70% of the study population had attained the homeostatic range of retinol after the supplementation. Therefore, alternate methods such as dose–response tests and isotope dilution are suggested to be utilised in future VAD-related studies; however, this is unlikely to be conducted in the vulnerable group of children with large-scale capacity and may give rise to ethical concerns [56, 57]. Nevertheless, the inclusion of extensive measurements of additional haematological, iron and inflammatory indicators has provided a more holistic evaluation of the schoolchildren's overall health improvement. Besides, the dropout rate of the current study (20.4%) was relatively low as compared to the anticipated dropout rate (40%) for rural schools, indicating a high level of compliance and acceptance among the schoolchildren regarding the consumption of RPO-enriched biscuits. Although dietary intake data had been collected at baseline in the present study, the data were found to be unreliable due to the prevailing illiteracy among the parents living in the rural areas, with statistical analysis detecting serious overestimation in the calorie intake which did not reciprocate with the physical measurement of schoolchildren with VAD [58]. In future, the inclusion of the assessment of hygiene practice and dietary change would be valuable as a possible explanation for the improved retinol outcome in both groups after supplementation.

Conclusion

The supplementation of RPO-enriched biscuits for a duration of 6 months was found to improve the overall health status of schoolchildren with VAD based on their blood biochemical status. Both retinol and RBP levels improved significantly in both groups, especially those with confirmed VAD; however, the improvements did not differ significantly between groups as a majority of them had reached the homeostatic range of retinol. Our findings indicate that RPO-enriched biscuits as a good source of bioavailable α- and β-carotenes, suggesting a bioconversion potential to vitamin A especially under nutritional stress. The 6-month supplementation of RPO-enriched biscuits was found to potentially enhance the iron status, which concomitantly promotes erythropoiesis. Supplementation of RPO-enriched biscuits has also been shown to exhibit potential anti-inflammatory effects, which may be attributed to its high phytonutrient contents. The potential health benefits of RPO are demonstrated in this study, rendering it a potentially viable food fortificant to be integrated into the National Nutritional Intervention Programmes to improve health status among vitamin A-deficient or malnourished children.

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Author contributions RL, KTT, YALL and SCL contributed to the design of this study. RL, KTT and KRS were involved in the funding acquisition. PYT and SNMJ were involved in the data collection. PYT performed the data analyses and wrote the manuscript. PYT, RL, KTT, YALL, SCL, RN and KRS reviewed and edited the manuscript. All the authors read, revised critically and approved the final manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding authors, RL and YALL. The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Declarations

Conflict of interest P.Y.T, K.T.T., R.L. and K.R.S are employees of the Malaysian Palm Oil Board, which received and managed grant RMK-11 (Eleventh Malaysia Plan) Grant-PD219/16 (public funding) and conducted the study. The other authors declare no conflict of interest.

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