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SHORT COMMUNICATION

Influence of *Spirulina* sp. and citric acid dietary supplements on the growth performance and immune parameters of common carp (*Cyprinus carpio*)

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Abstract This study explores the effects of supplementing the diets of common carp (*Cyprinus carpio*) with *Spirulina* sp. and citric acid (CA) on growth performance and immune parameters. Various experimental diets were formulated, including a control group, diets with 20g/kg Spirulina, 30g/kg Spirulina, 0.5g/kg citric acid, 1.0g/kg citric acid, and four mixed combinations of Spirulina and citric acid. Initially, common carp weighing 15.3 ± 1.9g were fed these diets for eight weeks in 40-L aquaria with three replicates. Growth performance and immune indices, specifically lysozyme, IgM, and immunoglobulin M (IgM) levels were assessed. The results demonstrated that the Spirulina/citric acid mixture significantly improved growth performance and immune indices compared to individual Spirulina and citric acid treatments and the control group ($P< 0.05$). The growth indices indicated increased dietary efficiency with the higher inclusion of the mixture, with the 30g *Spirulina* sp. + 1.0g citric acid and 30g *Spirulina* sp. + 0.5g citric acid treatments exhibiting the highest growth performance and the lowest feed conversion ratio (FCR). In conclusion, dietary supplementation of 30g/kg Spirulina combined with 0.5g/kg citric acid was found to promote growth and positively influence the immune parameters in common carp.

Keywords *Spirulina sp.* . *Cyprinus carpio* . Growth . Immune . Digestive enzymes

Introduction

Aquaculture has witnessed remarkable growth in animal protein production in recent decades. Among the most frequently cultivated fish worldwide is the common carp (*C. carpio*). This fish holds a significant position in the aquaculture industry of many Asian and some European countries. Notably, it is a valuable source of nutrients, making it a key component of healthy human diets. The common carp is highly prized for its numerous desirable traits, including rapid growth, efficient conversion of natural and supplementary feeds, and relative resilience to adverse environmental conditions and diseases. Proper nutrition is essential to ensure this cultivated fish's high survivability and accelerated growth rate.

Algae have recently emerged as pivotal food sources and additives in the commercial rearing of various aquatic animals, mainly fish and penaeid prawn larvae (Borowitzka 1997; Belay et al. 1996; Khatoon et

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al. 2010). Multiple studies have evaluated the nutritional value of dried microalgae as feed components for crustaceans and fish larvae (Biedenbach et al. 1990; Navarro and Sarasquete 1998; Khatoon et al. 2009). Kumar et al. (2010) analyzed the impact of periphyton and supplementary nutrition, using commercial pelleted feeds, on the growth performance of juvenile Nile tilapia *Oreochromis niloticus*. This lower plant group contains many vitamins, pigments, and nearly all essential nutrients, including polyunsaturated fatty acids (PUFA). It also serves as a valuable source of proteins and carbohydrates. Given the profound influence of various algae in general on the growth and vitality of fish, numerous algae species have been confirmed over time. Algae genera such as *Spirulina* sp. are widely embraced in aquaculture feeds due to their remarkable nutritional content (Avron and Ben-Amotz 1992; Lee 1997; Yamaguchi 1997).

Spirulina, in particular, has gained recognition as a valuable supplementary ingredient in aqua feed owing to its high protein and vitamin content. Microalgae typically boast a crude protein content of approximately 50%, with an amino acid profile akin to fishmeal. Furthermore, they serve as a source of polyunsaturated fatty acids (PUFAs), particularly those from the n-3 series. Consequently, microalgae have the potential to replace fish oil and fish meal in diets, enhancing meat quality through the deposition of n-3 PUFAs (Roy and Pal 2015; Sarker et al. 2016). Microalgae, known for their oil and molecule content, can also be harnessed as feed stocks for producing biofuels and high-value products, representing a promising renewable energy source (Moreno-Garcia et al. 2017). Spirulina, a commonly cultivated microalgae species in the commercial sector (Priyadarshani and Rath 2012; Farag et al. 2016), is a type of cyanobacteria renowned for its high protein content, ranging from 56% to 69% by dry weight. Additionally, Spirulina is replete with various bioactive compounds, including minerals, vitamins, carotenes, essential fatty acids, and antioxidants (Venkataraman 1997). Moreover, it serves as an effective substitute for animal-derived proteins in the diets of fish species, demonstrating favorable outcomes across a spectrum of species such as *Oreochromis niloticus* (Lu and Takeuchi 2004), *Paralichthys olivaceus* (Kim et al. 2015), *Clarias batrachus* (Dar et al. 2014), *Oplegnathus fasciatus* (Rahimnejad 2013), *Cirrhinus mrigala* (James et al. 2009), and *Poecilia reticulata* (Dernekbasi et al. 2010).

A significant portion of phosphate (P), roughly 60% to 70%, in vegetable protein ingredients is bound to citric acid. This binding may hinder the availability of P when there is an increase in dietary vegetable protein compounds and minerals like zinc, magnesium, and calcium (Denstadli et al. 2010). Adding citric acid (CA) to diets has enhanced P release from phytate in vitro (Zyla et al. 1995). Lowering the intestinal pH increases the solubility of P and phytate, thereby enhancing P absorption in the small intestine (Cross et al. 1990). Furthermore, apart from its impact on intestinal pH, the interaction between supplementary organic acids and various cations along the intestine can serve as a chelating agent, resulting in improved intestinal mineral absorption (Wood and Serfaty-Lacrosniere 1992). While extensive research exists regarding the effect of dietary acidification on mineral utilization in terrestrial animals, studies involving fish have been somewhat limited (Sarker et al. 2005). Experiments conducted with pigs have indicated that CA supplementation promotes growth performance (Sugiura et al. 2001). In the case of rainbow trout, citric acid supplementation in their diet has been observed to chelate CA and P, increasing their solubility and enhancing mineral utilization (Vielma et al. 1999).

Therefore, microalgae can be directly employed as a live culture or a value-added dietary supplement. It is imperative to recognize that dietary patterns significantly influence the development and functionality of the immune system. Hence, any newly formulated algae diet must meet the specific nutritional requirements of the fish. In the present investigation, Spirulina algae and citric acid were isolated, blended at varying levels, and assessed over 8 weeks to discern which combinations yielded superior growth rates and immune system performance.

Materials and methods

Fish and experimental procedure

This study was conducted at the Research and Education Center for Natural Resources and Agriculture at the Fisheries Campus in Guilan Province, Iran. The adaptation process for the fish involved housing them in a 2000-liter tank and providing them with the prescribed dietary regimen for one week. Following this acclimatization, the fish were relocated to twenty-seven 40-liter aquaria equipped with central aeration. To

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ensure the water quality remained suitable for the fish, physicochemical parameters were monitored using a dial oximeter (OXI3230B/SET) and a pH meter (PH330i/SET), with measurements taken three times daily.

Experimental design, diets, and performance

A commercial carp feed supplied by Dan-Vahdat Co., Iran, was employed in the experiments. The feed consisted of 35% crude protein and included wheat flour, wheat bran, fish meal, soybean meal, corn, and rice bran. Additionally, the feed was supplemented with Spirulina and citric acid.

Spirulina powder and citric acid were evenly distributed onto the feed and dried at ambient temperature. Analysis of the fish diet revealed the following nutritional content: crude protein at 35%, fat at 11%, fiber at 10%, ash at 14%, calcium at 6%, phosphorus at 9%, and moisture at 14%. The ingredients included in the fish meal were corn meal, soybean meal, corn gluten, soy protein, concentrated wheat meal, soybean oil, fish meal, sodium chloride, choline chloride, vitamin and mineral premixes, probiotics, amino acids, and antioxidants.

The experimental methodology encompassed a control group and several treatment groups, each denoted by specific quantities of Spirulina powder and citric acid per kilogram of diet. These treatments were as follows: 0g of Spirulina powder and 0g of citric acid per kilogram of diet (SpCA-0), 20g of *Spirulina* (Sp20), 30g of *Spirulina* (Sp30), 0.5g of citric acid (CA0.5), 1g of citric acid (CA1), 20g of *Spirulina* + 0.5g of citric acid (Sp20+CA0.5), 20g of *Spirulina* + 1g of citric acid (Sp20+CA1), 30g of *Spirulina* + 0.5g of citric acid (Sp30+CA0.5), and 30g of *Spirulina* + 1g of citric acid (Sp30+CA1) per kilogram.

The fish received two daily feedings, and biometric measurements were taken at the beginning, middle, and end of the experimental period, with a 24-hour interval after feeding cessation. Randomly selected fish had blood samples collected and transferred to the hematology laboratory for immunological assessment. A comprehensive database was maintained, with monthly biometric measurements, including fish body length and weight data.

Measurement of growth performance

Statistical calculations on growth indices and food efficiency were conducted based on the following formulas:

BWI (*Body weight* index%) = 100% (Wt-W0) / W0 [W0 = Mean initial weight (g); Wt = Average final weight (g)]

FCR (Food conversion ratio) = F / (Wt-W0) [F = the amount of food consumed by fish; W_0 = Mean primary biomass (g); W_t = Mean final biomass (g)]

SGR (Specific growth rate) = (ln Wt -lnW0) / t × 100 [W0 = Mean primary biomass (g); W_t = Mean final biomass (g); T = Period (days); *ln* is the natural log, and *t* stands for the experimental period in days]

Immunological factors

The investigation of immunological factors was conducted during the final phase of this research, which took place in the eighth week. Blood samples from the fish were randomly collected for analysis. To ensure a stress-free process, the fish were initially anesthetized using clove powder, and their blood was then extracted from the caudal vasculature using insulin syringes. A total of ten blood specimens were obtained from each replicate group. The exact number of samples was collected from the treatment and control groups, each with three replicates. Subsequently, the blood samples were processed to obtain blood serum through centrifugation.

The Immunoglobulin M (IgM) levels were quantified using the immunoturbidimetric method. In this method, IgM forms a complex with polyclonal antibodies in buffer solutions, causing the solution to become turbid. The degree of turbidity is directly proportional to the concentration of IgM and was measured using a spectrophotometer, with distilled water serving as the blank (Model 2100-VIS by Unico USA) at a wavelength of 340 nm. The determination of serum lysozyme levels involved utilizing 1.75 ml of micrococcus lysodeikticus (Sigma) suspension, equivalent to 0.375 mg/ml sodium phosphate buffer at 0.05 M and pH 6.2. To this suspension, 250 microliters of mixed serum samples were added, and light absorption

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readings were taken after 15 and 180 seconds using a spectrophotometer at a wavelength of 670 nm. A blank was established using sodium phosphate buffer. The quantification of total immunoglobulin concentrations was performed according to the methodology delineated by Siwicki and Anderson in 1993 and the approach outlined by Amar et al. in 2000. In brief, the serum sample underwent the Biuret method. More specifically, 0.1 ml of each serum sample was mixed with 0.1 ml of a 32% polyethylene glycol solution (PEG, 10,000 MW, Sigma Chemical, St. Louis, MO, USA). This resultant mixture was subsequently incubated for 2 hours, facilitating the precipitation of immunoglobulin molecules. The precipitated immunoglobulin was separated through centrifugation (Eppendorf Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) at 5000 revolutions per minute and a temperature of 4°C. The supernatant fluid was subjected to measurement for total protein content, and the immunoglobulin concentration was computed employing the subsequent formula: Total Immunoglobulin Concentration (mg per ml) = Total protein treated with polyethylene glycol - Total protein in serum samples. This approach allowed us to determine the immunoglobulin concentration in milligrams per milliliter.

Data analysis

The normality of the data was evaluated utilizing the Shapiro-Wilk test. To ascertain the significance of the experiments, a one-way ANOVA analysis was conducted employing the Duncan method at a 95% confidence level ($P< 0.05$). The data analysis was executed using SPSS 20.0 and Excel 2010 software.

Results

Growth parameters analysis

The study outcomes reveal distinct daily growth rates among various treatment groups. Notably, the highest daily growth rate was observed in SP30+CA0.5 (0.13 ± 0.06) and SP30+CA1 (0.13 ± 0.03), while the lowest was associated with SP20 (0.08 ± 0.01). Statistical analysis demonstrates significant variance between the treatments throughout the experiment ($P <0.05$) (Table 1). Discriminant testing further indicates a significant divergence in the daily growth coefficient of SP30+CA0.5 compared to other treatments. However, it exhibited no statistical distinctions from SP20+CA0.5, SP20+CA1, and SP30+CA1 ($P >0.05$). Additionally, the results suggest that SP20, SP30, CA0.5, and CA1 share a statistically equivalent daily growth coefficient ($P >0.05$).

Findings show considerable differences in weight gain rates among the treatment groups. Specifically, the highest weight gain rate was observed in SP30+CA1 (8.7 ± 0.31), whereas the lowest was associated with SP20 (4.76 ± 0.61) (Table 1). Statistical assessments reveal significant differences in weight gain among the treatments during the experimental period ($P <0.05$). Discriminant testing indicates a noteworthy difference in the weight gain of SP30+CA1 compared to other treatments. Nonetheless, SP30+CA1 did not display a statistically significant difference from SP30, SP20+CA0.5, SP20+CA1, and SP30+CA0.5 ($P >0.05$). Furthermore, results indicate that SP20, CA0.5, and CA1 statistically share the same weight gain ($P <0.05$).

The study demonstrates variations in the percentage of body weight index among the treatment groups. The highest percentage of body weight index is associated with SP30+CA1 (35 ± 91 ± 1.47), while the lowest is related to SP20 (21.22 ± 2.98) (Table 1). Statistical analysis indicates significant differences in body weight index among the treatments during the experimental period ($P <0.05$). SP30+CA1, however, does not exhibit any significant differences from SP20+CA1 and SP30+CA0.5 ($P >0.05$). Results further reveal that SP20, SP30, CA0.5, and CA1 statistically share the same percentage of body weight index ($P >0.05$).

The investigation unveils varying specific growth coefficients among the treatment groups. Specifically, the highest specific growth coefficient is associated with SP30+CA1 (0.178 ± 5.11), while the lowest is linked to SP20 (0.40 ± 3.2) (Table 1). Statistical tests demonstrate significant differences in specific growth rates among the treatments throughout the experiment ($P <0.05$). According to the findings, the specific growth coefficient of SP30+CA1 significantly differs from that of other treatments ($P <0.05$). However, no statistical differences are observed with SP20+CA0.5, SP20+CA1, and SP30+CA0.5 ($P >0.05$). Additionally, results indicate that SP20, SP30, CA0.5, and CA1 share the same specific growth rate (Table. 1).

Table 1 Effect of Spirulina and CA supplementation on growth performance of common carp in different treatments

		Spirulina and CA concentrations g per kg (food)							
	Control	*Spirulina*		CA		Mixture (Spirulina+CA)			
		20	30	0.5	1.0	20+0.5	20+1.0	30+0.5	30+1.0
$W0_{(g)}$	22.20±3.70[a*]	22.40±6.28[a]	22.60±5.36[a]	24.00±4.83[a]	22.90±6.10[a]	23.60±5.13[a]	22.70±8.10[a]	22.80±5.1[a]	22.70±6.38[a]
$Wt_{(g)}$	24.00±8.34[a]	25.60±7.72[a]	26.38±4.57[a]	23.80±3.61[a]	26.25±2.95[a]	25.10±4.95[a]	27.50±11.34[a]	25.44±6.39[a]	26.44±5.43[a]
$GR_{(g)}$	0.08±0.01[b]	0.08±0.01[b]	0. 10±0.01[bc]	0.09±0.01[cd]	0.10±0.01[cd]	0.12±0.01[ab]	0.12±0.01[a]	0.13±0.01[a]	0.13±0.01[a]
$WG_{(g)}$	4.82±0.59[b]	4.76±1.05[b]	6.08±0.78[bc]	5.15±0.07[cd]	5.79±0.52[cd]	6.98±0.50[ab]	7.51±0.49[a]	7.70±0.73[a]	8.06±0.53[a]
BW%	21.65±2.83[cd]	21.22±4.98[d]	27.07±3.77[bc]	23.21±0.40[cd]	25.94±2.08[cd]	31.52±2.27[ab]	33.45±2.50[a]	34.47±3.71[a]	36.91±2.55[a]
SGR%	3.26±0.39[cd]	3.20±0.70[d]	3.99±0.49[bc]	3.48±0.06[cd]	3.84±0.28[bcd]	4.56±0.28[ab]	4.81±0.31[a]	4.93±0.47[a]	5.11±0.31[a]
K%	1.85±0.30	1.93±0.42	1.91±0.43	1.83±0.36	1.85±0.39	1.88±0.41	1.89±0.37	1.86±0.38	1.98±0.45
FCR	3.14±0.39[ab]	3.28±0.83[a]	2.49±0.31[cde]	2.91±0.05[abc]	2.60±0.23[bcd]	2.15±0.15[de]	2.00±0.13[de]	1.96±0.19[de]	1.86±0.12[e]

*a-d showed differences between treatments by Duncans test.

Table 2 Effects of Spirulina and citric acid (CA) supplementation on immunological indices of common carp in different treatments

	Control	Sp20	Sp30	CA0.5	CA1	Sp20+CA0.5	Sp20+CA1	Sp30+CA0.5	Sp30+CA1
Lysozim(u/ml/min)	30.11±2.06[a*]	29.33±2.08[a]	27.33±2.52	30.33±2.08 [b]	29.00±1.00[b]	28.00±2.00[b]	26.00±3.46[b]	32.33±3.06[b]	33.00±1.73[b]
Igm(mg/dl)	31.28±3.95[a]	32.33±4.93[a]	41.00±5.29[a]	48.67±4.04[c]	41.33±1.53[b]	41.00±3.61[b]	41.67±3.06[b]	46.67±2.52[bc]	40.67±1.53[b]
Imonoglobin(mg/ml)	14.06±0.27[a]	15.23±0.31[ab]	15.97±0.32[a]	16.67±0.15[d]	16.43±0.15[d]	15.17±0.32[b]	15.63±0.15[bc]	17.20±0.30[c]	16.63±0.15[d]

*a-d showed differences between treatments by Duncans test.

The study reveals variations in the feed conversion ratios among the treatment groups. The highest recorded feed conversion ratio belongs to (0.48 ± 3.28), whereas the lowest level is associated with SP30+-CA1 (0.07 ± 1.86). Statistical tests indicate significant differences in feed conversion ratios among the treatments throughout the experiment ($P < 0.05$). Based on the separation test, the feed conversion ratio of SP20 significantly differs from other treatments ($P < 0.05$). However, it does not exhibit statistically significant differences from SP30, CA0.5, and CA1. Meanwhile, results show that SP20+CA0.5, SP20+CA1, and SP30+CA0.5 share the same feed conversion ratio ($P > 0.05$).

Immunological findings

The results of this experiment revealed that the highest lysozyme levels were observed in conjunction with Sp30+CA0.5 and Sp30+CA1, while the lowest levels were recorded for the Sp20 treatment (Table 2). A statistical analysis demonstrated a significant variance among the treatments ($P < 0.05$). Additionally, it was observed that Sp30, CA0.5, CA1, Sp20+CA0.5, and Sp20+CA1 exhibited comparable lysozyme levels with no statistically significant distinctions ($P > 0.05$).

According to the results, the highest IGM concentrations were found in specimens exposed to Sp30+-CA0.5 and CA0.5, while the lowest concentrations were detected in those treated with Sp20 (Table 2). The statistical tests confirmed a significant distinction among the treatments ($P < 0.05$). Furthermore, the findings indicated that Sp30, CA1, Sp20+CA0.5, and Sp20+CA1 exhibited equivalent IGM levels with no significant discrepancies ($P > 0.05$).

The study's outcomes demonstrated that Sp30+CA0.5 displayed the highest immunoglobulin M content, whereas the lowest was associated with the Sp20 treatment. A one-way analysis of variance test revealed a statistically significant distinction between Sp30+CA0.5 and Sp20 regarding immunoglobulin M levels ($P < 0.05$). Moreover, Sp30, CA0.5, CA1, Sp20+CA0.5, and Sp20+CA1 exhibited similar immunoglobulin M content (Table 2).

Discussion

In recent years, there has been a concerted effort to replace fish meal, partially or entirely, with various plant-based sources capable of providing nutritionally dense and valuable feed. Spirulina powder, specifically *Spirulina platensis*, has emerged as a promising candidate for future aquaculture practices (Lu and Takeuchi 2004; Choonawala 2007). Due to its high protein content and essential amino acids, fish meal remains a crucial component in aquatic feeds (Güroy et al. 2022; Yousefi et al. 2022). According to the findings of this research, the growth parameters exhibited a more favorable response when higher concentrations of Spirulina and citric acid were introduced during the grow-out phase of the sample. During the initial sampling stage, Sp20+CA0.5 demonstrated the highest growth indices at the end of the eighth week. Sp30+CA0.5 and Sp30+CA1 exhibited the highest body weight index, weight gain, specific growth rate, and the lowest feed conversion ratio. Fat accumulation exceeded 15% in different concentrations, but fat levels increased with the increased inclusion of Spirulina and citric acid in Sp30+CA0.5 and Sp30+CA1. Previous research indicated that dietary lipid levels above 15% adversely affected growth and food intake in species like common carp (Jauncey 1979). In contrast, the maintenance of optimal lipid levels has been shown to yield a multitude of benefits in aquaculture, including enhanced growth rates, improved feed conversion efficiency, efficient nutrient utilization, and reduced nitrogen excretion, as demonstrated in previous studies (Yigit et al. 2002; Martin et al. 2007). The present research reinforces the significance of sustaining optimal lipid content across all treatment groups. Notably, this study highlights the promising outcomes achieved by fish groups fed with varying concentrations of Spirulina and citric acid, specifically the SP30+-CA0.5 (3% Spirulina + 0.5g of citric acid per kg of diet) and SP30+CA1 (3% Spirulina + 1g of citric acid per kg of diet) treatments, which exhibited superior feed conversion efficiency and growth performance. These findings underscore the potential of nutritionally rich algae as a valuable source of fish feed, in line with the observations made by Khatoon et al. (2010). Various factors, including diet acceptability, have been recognized for their influence on the growth performance of fish species, as previously observed in catfish (*Clarius batrachus*) by Hasan et al. (1989). The current study aligns with the notion that diets incorporating algae protein can significantly enhance growth performance compared to control diets.

In the context of the present investigation, it was found that lysozyme, immunoglobulin, and Immunoglobulin M (IgM) levels exhibited notable increases, with the highest levels observed in the SP30+CA0.5 treatment group. Lysozyme activity in fish blood plays a pivotal role in assessing the innate immune system, as acknowledged by Montoya et al. (2017) and Amphan et al. (2019). Research by Reda et al. (2018) also documented increased enzyme activity in tilapia following dietary supplementation with yeast nucleotides. Furthermore, the supplementation of probiotic *Bacillus subtilis* endospores has been shown to enhance lysozyme activity in tilapias, as reported by Galagarza et al. (2018), Faheem et al. (2022), and Rosenau et al. (2022).

Numerous factors, both internal and external, can exert an influence on parameters related to the innate immune response in fish. Temperature fluctuations, stress management, and stocking density are known to have suppressive effects on these responses, while certain food additives and immunostimulants have the potential to enhance their efficiency, as documented by Magnadottir in her studies from 2006 and 2010 (Magnadottir 2006, 2010). Changes in environmental pH levels have yielded mixed results regarding immune system parameters, including lysozyme and IgM levels in the circulation. Notably, the absence of stressors in the experimental fish samples, attributed to the adequate concentrations of Spirulina and citric acid, is reflected in the heightened levels of immune-related factors.

The dietary supplementation of Spirulina and citric acid, particularly in the SP30+CA0.5 treatment, emerges as a strategy that enhances immune responses and augments disease resistance, particularly in challenging environmental conditions. Recent research corroborates these findings, demonstrating the up-regulation of immune-related genes in rainbow trout when exposed to diets containing Spirulina (Güroy et al. 2022; Yousefi et al. 2022).

Conclusion

Based on the findings of this study, it is evident that a combination of Spirulina and citric acid can exert a more significant influence on weight gain, growth performance, and immune system enhancement. Consequently, Spirulina and citric acid can potentially enhance nutrient levels by substituting protein content. Additionally, citric acid contributes to improved absorption of vegetable protein, thereby promoting growth indices. Furthermore, incorporating Spirulina and citric acid into a fish diet can induce notable changes in various immunological factors in fish, with the most pronounced effects observed in the SP30+CA0.5 combination, leading to enhanced immune responses.

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Conflicts of interest The authors declare no conflict of interest.

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