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Immune response of patients with recurrent aphthous stomatitis challenged with a symbiotic

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Background: There are indications that Th1 polarization of immune response plays an important role in the pathogenesis of recurrent aphthous stomatitis (RAS), and that the use of probiotics can stimulate immune regulatory activity, influencing the course of the disease. The aim of this study was to characterize the initial immune profile of RAS patients and evaluate clinical and serological response following a challenge with symbiotic treatment containing fructooligosaccharide, *Lactobacillus*, and *Bifidobacterium*.

Methods: The immune responses of the 45 patients with RAS, submitted to symbiotic or placebo for 120 days, in relation to 30 RAS-free controls, were evaluated over a period of 6 months. Peripheral blood was collected from all patients at 0 (T0), 120 (T4), and 180 days (T6) after the start of treatment and Th1 (IL12-p70, IFN-γ), Th2 (IL-4), Treg (IL-10), Th17 (IL-17A), inflammatory (TNF-α, IL-6)-associated cytokines, and clinical parameters were quantified.

Results: At T0, significant differences were found in the serological levels of the IFN-γ, IL-4, and IL-6 cytokines of the RAS patients in comparison with the controls. It was observed that the cytokine profile of the RAS group was comprised of 2 distinct clusters: a pure Th2 and a Mixed (Th1/Th2) subtype and that symbiotic treatment induced an improvement in pain and an increase in IFN-γ levels, producing a reduction in Th2 response.

Conclusions: In RAS, symbiotic treatment based on a fructooligosaccharide, Lactobacillus, and Bifidobacterium composition produced an alteration in the Th2 serological immune profile in the direction of Th1 and improved pain symptomatology.

KEYWORDS
cytokines, immunology, recurrent aphthous stomatitis, treatment

1 | INTRODUCTION

Recurrent aphthous stomatitis (RAS) is a chronic oral inflammatory disease that remains a clinical problem without a solution. Although the etiopathogenesis of RAS is still unknown, the disease is considered to be multifactorial. Some studies suggest that immune system dysfunction may be one of the main factors responsible for the occurrence of RAS, but a specific defect has not yet been discovered.[1,2] The association between the aggravation or improvement of RAS and changes in the equilibrium of Th1/Th2 activation suggests the existence of Th1 hyperimmune responsiveness in patients with RAS. It was recently proposed that imbalance between the Th1 and Th2 immune responses may contribute to a loss of immunological tolerance in the oral mucosa, favoring the occurrence of inflammatory reactions, and the appearance of ulcers. The local and systemic predominance of Th1 cytokines in RAS has been reported by several studies.[2-4]

Although the lack of understanding of the etiopathogenesis of RAS has prompted several research studies, no curative treatment

for the disease has been described to date, and only palliative or symptomatic approaches have been used clinically. Probiotics, meanwhile, are living commensal microorganisms that are beneficial for health when present in appropriate amounts and can be used to modulate an exacerbated immune response. Evidence indicates that certain types of probiotics stimulate the activity of Treg lymphocytes and inhibit Th1 or Th2 immune responses.[5-7] In addition, probiotic therapy could be improved by the use of prebiotics such as oligosaccharides, inulin, and lactosucrose, whose combination is known as symbiotic.[8]

There are indications that the polarization of the immune system for Th1 responses has an important role in the pathogenesis of RAS and that the use of symbiotics could stimulate the activity of regulatory T lymphocytes which would favorably influence the evolution of RAS. From this perspective, the aim of this study was to characterize the initial immune profile of RAS patients and evaluate serological immune response during and after treatment with a symbiotic compound containing fructooligosaccharide, *Lactobacillus*, and *Bifidobacterium*.

2 | METHODS

2.1 | Participants

Individuals were recruited from the Stomatology department of the Federal University of São Paulo, Brazil (UNIFESP), after signing the informed consent form approved by the Ethics and Research Committee. The participants consisted of 90 volunteers, of whom 60 were patients with RAS and 30 were healthy individuals. The following inclusion criteria were used as follows: age between 18 and 60, male or female, with a history of recurrent oral ulcers for at least 12 months, with at least 1 occurrence per month. The exclusion criteria were as follows: alcohol and tobacco use, pregnancy, previous or current malignant neoplasms and their respective treatments, autoimmune diseases, general immune deficiencies, Crohn's Disease, Behcet's Disease, Reiter's Syndrome, and cyclical neutropenia.

The RAS patients were randomly divided into 2 groups of 30 patients each, using the computer software. One of the groups was submitted to a placebo (Placebo) while the other underwent symbiotic treatment (Symbiotic) for 120 days, following a double-blind experimental design. The symbiotic and placebo products were in the form of sachets, with the symbiotic envelope contained 6 g of fructooligosaccharide and the following strains in powder form: 10^8-10^9 Colony Forming Units (CFU) of *Lactobacillus paracasei* Lpc-37 SD 5275, 10^8-10^9 CFU of *Lactobacillus rhamnosus* HN001 SD 5675, 10^8-10^9 CFU of *Lactobacillus acidophilus* NCFM SD 5221, and 10^8-10^9 CFU of *Bifidobacterium lactis* HN019 SD 5674; while the placebo prepared with the inert compound maltodextrin. The sachets were prepared by a pharmaceutical laboratory in such a way as to make it impossible for the patient and clinician to know which treatment was being used until the study was over, and the sachet code was revealed.

In the first consultation, a clinical exam and characterization of the RAS were carried out. The patients were instructed to ingest 1 sachet twice a day: in the morning and in the evening after meals, for 120 consecutive days. The patients were monitored for 60 additional days after the interruption of treatment. All participants were also instructed to return the empty sachets at their monthly appointments.

Peripheral blood samples were collected from all patients before treatment at 0 (T0), at 120 (T4) and 180 days (T6) following the start of treatment. Samples were collected from the patients of the healthy group (n = 30) for comparison with the RAS group (n = 60) at T0 only. The blood withdrawal from RAS patients was scheduled outside outbreak periods when they were free of lesions. In accordance with ethical principles, RAS patients could use local steroids during outbreaks. In such cases, however, the blood withdrawal was performed after an interval of 1 week from topical medication use. Additional blood tests did not be performed to rule out hematinic deficiencies as an underlying cause for an aphthous like ulcers.

2.2 | Clinical evaluation

Patients received a notebook in which they were asked to register the presence and the number of lesions, the day that they appeared and disappeared, a visual analysis scale (VAS) value for pain, and the size of the ulcers, measured by an endodontic ruler.

2.3 | Immunological profile evaluation

Blood collection was performed in the morning between 8 and 10 h using Vacutainer® tubes without anticoagulants. Blood samples were centrifuged, and the cell-free serum was taken out, and stored at −20°C for the quantification of cytokines when all samples were collected.

2.4 | Quantification of cytokines

Th1 (IL12-p70, IFN-γ), Th2 (IL-4), Treg (IL-10), Th17 (IL-17), and inflammatory cytokines (TNF-α, IL-6) were quantified in the RAS (at T0, T4, and T6) and control groups (at T0), using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel kit (HCYTOMAG-60K, Millipore Corp., Billerica, MA, USA) according to the manufacturer's recommendations.

2.5 | Immune profiles of patients and volunteers

The cytokines levels were used to classify the immune profile of the participants. Cytokine values lower than the Median+3*Qn^{ct} (robust scale estimator of deviation of cytokine values from control group) threshold were considered low, while cytokine values higher than the Median+3*Qn^{ct} threshold were considered high.[9] The Qn^{ct} threshold corresponds to the range of values reported by 99% of the control population. The immune profile was classified in accordance with the Table 2.

2.6 | Statistical analysis

Analysis of variance (ANOVA) with repeated measures, Tukey's test, Levene's test, Student's *t* test, Mann-Whitney *U* test, exact test of Fisher, and Ordinal Regression Logistic was used for the statistical analysis of the results. Statistical tests were performed using SPSS version 10.0 (IBM Corp., Armonk, NY, USA), and R (www.r-project.org) and *P*-values < .05 were considered significant.

3 | RESULTS

The study began with 90 patients, of which 30 were healthy individuals (control group) and 60 were RAS carriers. Among the 60 original RAS patients, only 45 remained in the study until the end. When epidemiologic characteristics (mean age, age range, gender, race/ethnicity) were compared, no significant difference was identified between control and RAS groups (Table 1). Considering the clinical profile of RAS patients submitted to symbiotic or placebo treatment, it was observed that there was no significant difference in the clinical characteristics (RAS type, family history, triggers factors: stress, trauma, and food) or in the frequency of use of topical steroids during outbreaks between the 2 groups (Table 1).

3.1 | Serum cytokine levels from 2 groups (control and RAS) measured before the start of treatment

With respect to the immunological profiles of the patients, a significant difference was observed in the distribution of the cytokine levels between the Control and RAS groups. The proportion of patients with outlier levels of IFN-γ (20% in RAS vs 0% in control; $P < .05$), IL-4 (64% in RAS vs 0% in control; $P < .01$), and IL-6 (16% in RAS vs 0% in control; $P < .05$) was significantly higher in the RAS group than in the control (Figure 1).

3.2 | Immune profiles of patients and volunteers measured before the start of the treatments

In the analysis of cytokine patterns at time point 0, the proportion of RAS patients classified as Th0 (33%; n = 15) was significantly ($P < .01$) lower than the control counterpart (87%; n = 26). Nevertheless, the proportions of RAS patients classified as Th2 (47%; n = 21; $P < .01$) or Mixed Th1/Th2 (18%; n = 8; $P < .05$) were

TABLE 1 Clinical characteristics of the participants of the study (n = 75)

			RAS (n = 45)		
Characteristics	**Parameter**	**Control (n = 30)**	**Symbiotic (n = 22)**	**Placebo (n = 23)**	***P***
Age (y)	Mean	37.4	36.6	38.3	.834[a]
	Range	23-58	19-57	23-58	.725[b]
Gender	Male	14 (49%)	14 (64%)	15 (65%)	.622[a]
	Female	16 (51%)	8 (36%)	8 (35%)	.841[b]
Ethnicity	White	24 (80%)	19 (87%)	22 (96%)	.328[a]
	Black	3 (10%)	2 (9%)	0 (0%)	.358[b]
	Mixed	3 (10%)	1 (4%)	1 (4%)	
Factors associated					
Family history	Yes		11 (50%)	12 (52%)	.887
	No		11 (50%)	11 (48%)	
Stress	Yes		11 (50%)	17 (74%)	.129
	No		11 (50%)	6 (26%)	
Trauma	Yes		6 (27%)	8 (35%)	.749
	No		16 (73%)	15 (65%)	
Food	Yes		13 (59%)	10 (43%)	.376
	No		9 (41%)	13 (57%)	
RAS type	Minor		20 (91%)	22 (96%)	.738
	Major		1 (4.5%)	1 (4%)	
	Herpetiform		1 (4.5%)	0 (0%)	
Steroid use in relation to total number of clinical appointments	Yes		8 (6.8%)	4 (3.0%)	.378
	No		130 (93.2%)	128 (97.0%)	

The characteristics were compared between Control and RAS[a] group and between Symbiotic and Placebo[b] using Student's *t* test and the exact Test of the Fisher.

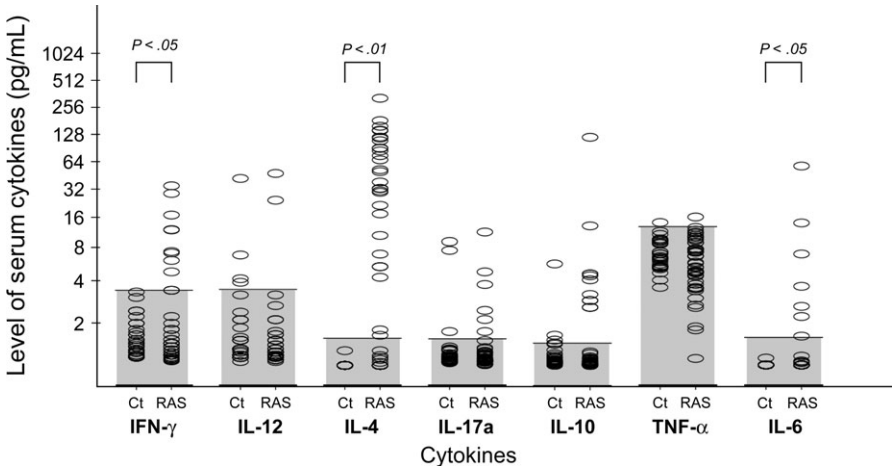

FIGURE 1 Scatter plot showing the distribution of serum cytokine levels from 2 groups (control and RAS) measured at the beginning of the research (time T0). The gray frame represents the upper limit of cytokine levels calculated using the median + 3 Qn dispersion interval of cytokines from control group, which corresponds to the range of values reported by 99% of the control population. The cases above the limits were considered outside the normal range. The comparison of the number of outliners between control and RAS group was executed using the exact test of Fisher. Qn = robust scale estimator proposed by Rousseeuw and Croux in 1993

significantly higher than in the control group (0%; n = 0). The results showed no significant difference in the patterns of the serum cytokines Th17/Treg or the anti-/pro-inflammatory cytokines between the 2 types of participants (Table 2).

3.3 | Difference of serum cytokines between time points T0, T4, and T6 from symbiotic and placebo groups

In factorial analysis (time, treatment, and interaction time × treatment) of the cytokine levels, a difference was found in the levels of INF-γ from the RAS group receiving symbiotic treatment at the T6 time point in comparison with the placebo ($P < .05$). There was also an increase in the IL-4 level in T4 in relation to T0 and T6 ($P < .05$), which was independent of the treatment and time × treatment factors. In relation to the other cytokines, we did not find any significant differences associated with the 2 main effects (treatment, time) or their interaction (Table 3).

3.4 | Immune response after 6 months of follow-up

When comparing the relative difference of the immune profile from RAS patients between the T4-T0 interval (Table 4), it was observed

TABLE 2 Immune serum profile before treatments (n = 75)

Immune profile	Serum profile	Control (n = 30)	RAS (n = 45)	P^a
Th1 (IFN-γ or IL-12) vs Th2 (IL-4)				
Th0	Low (IFN-γ and IL-12) and Low IL-4	26 (87%)	15 (33%)	<.01**
Th1	High (IFN-γ or IL12) and Low IL-4	4 (13%)	1 (2%)	.150
Th2	Low (IFN-γ and IL12) and High IL-4	0 (0%)	21 (47%)	<.01**
Mix (Th1/Th2)	High (IFN-γ or IL12) and High IL-4	0 (0%)	8 (18%)	<.05*
Treg (IL-10) vs Th17 (IL-17A)				
Treg(−) and Th17(−)	Low IL-10 and Low IL-17	24 (80%)	30 (67%)	.295
Treg > Th17	High IL-10 and Low IL-17	3 (10%)	9 (20%)	.341
Treg = Th17	High IL-10 and High IL-17	1 (3%)	2 (4%)	1.000
Treg < Th17	Low IL-10 and High IL-17	2 (7%)	4 (9%)	1.000
Pro-inflammatory (TNF-α or IL-6) vs anti-inflammatory (IL-10)				
Infl(−) and anti-infl(−)	Low (TNF-α and IL-6) and Low IL-10	26 (87%)	32 (71%)	.1624
Inflammatory	High (TNF-α or IL-6) and Low IL-10	0 (0.0%)	2 (4%)	.513
Anti-Inflammatory	Low (TNF-α and IL-6) and High IL-10	3 (10%)	5 (11%)	1.000
Mix	High (TNF-α or IL-6) and High IL-10	1 (3%)	6 (13%)	.231

Low cytokine: cytokine < median +3*Qn^{ct}; high cytokine: cytokine > median +3*Qn^{ct} When,the referred median corresponds to the cytokine median of the control group and the Qn^{ct} correspond to cytokine robust scale estimator deviation of the control group.

[a]Fisher's exact test.

* and ** considered significantly different at 5% and 1% levels, respectively.

TABLE 3 Comparison of the difference of serum cytokines between time points T0, T4, and T6 of each patient from groups Symbiotic and Placebo (n = 45)

		Treatments				
Cytokines	Time (mo)	Placebo (Median ± Qn)	Symbiotic (Median ± Qn)	*P* treatment	*P* time	*P*. treatment × time
INF-γ	0	1.31 ± 0.958	1.15 ± 0.833	.434	.139	.027*
	4	1.15 ± 0.833	1.47 ± 1.175			
	6	1.31 ± 0.667	2.00 ± 1.572			
IL-12	0	0.87 ± 0.354	0.87 ± 0.474	.816	.855	.194
	4	0.87 ± 0.354	1.04 ± 0.644			
	6	0.70 ± 312	0.96 ± 0.549			
IL-4	0	0.94 ± 1.958	23.19 ± 31.96	.718	.037*	.913
	4	70.36 ± 60.98	56.05 ± 69.96			
	6	16.87 ± 22.92	36.14 ± 55.28			
IL-17	0	0.45 ± 0.542	0.42 ± 0.322	.755	.505	.457
	4	0.64 ± 0.458	0.37 ± 0.436			
	6	0.64 ± 0.604	0.54 ± 0.530			
IL-10	0	0.25 ± 0.249	0.38 ± 0.492	.315	.459	.210
	4	0.28 ± 0.354	0.57 ± 0.871			
	6	0.19 ± 0.167	0.38 ± 0.492			
TNF-α	0	6.21 ± 2.791	4.53 ± 3.600	.125	.544	.764
	4	6.03 ± 3.000	5.46 ± 2.993			
	6	5.93 ± 3.062	5.70 ± 3.107			
IL-6	0	0.00 ± 0.000	0.00 ± 0.000	.772	.837	.074
	4	0.00 ± 0.000	0.00 ± 0.000			
	6	0.00 ± 0.000	0.00 ± 0.000			

The comparison of the main effects and interactions was performed using the ANOVA for repeated measures after Z transformations of data and test of the normality. The expression values were represented by median ± Qn robust scale (equivalent to SD of normal distributions).

that symbiotic treatment significantly increased the proportion of patients with a Th0 immune profile (symbiotic: 18.2% vs placebo: 4.3%) and also inhibited its decline (symbiotic: 13.6% vs placebo: 43.5%). In addition, when comparing both intervals: T0-T4 and T0-T6, the symbiotic treatment reduced the proportion of patients in whom the immune profile changed to Th2 (T4-T0: symbiotic: 18.2% vs placebo: 47.8%; T6-T0: symbiotic: 9.1% vs placebo: 39.1%) and was also responsible for reducing the proportion of patients that maintained the Th2 profile (T4-T0: symbiotic: 22.7% vs placebo: 4.3%; T6-T0: symbiotic: 31.8% vs placebo: 17.4%; Table 4).

3.5 | Clinical evaluation during 6 months of follow-up

On the other hand, when analyzing the clinical parameters, it was observed that pain level was the only clinical variable that differed significantly ($P = .027$) between the RAS group receiving the symbiotic and the group submitted to placebo (Table 5). Moreover, the patients that used the symbiotic treatment had less pain than ones treated with the placebo after 6 months. The reduction of pain was independent of the initial immune profiles and the difference in the cytokine measurements between T4-T0 and T6-T0 (data not shown).

No significant differences were detected between the symbiotic treatment and the placebo groups in terms of total number of lesions (TNL; $P = .629$), average healing time (AHT; $P = .444$), maximum lesion size (MLS; $P = .407$), and number of outbreaks (NO; $P = .512$). Considering the difference between the time points in both groups, total number of lesions, pain, average healing time, maximum lesion size, and total numbers of outbreaks, decreased between T0 and T4 and remained stable between T4 and T6 (Table 5). In relation to reported side effects, there were significant differences in the rates of flatulence ($P = .003$) and loose bowels ($P = .04$) between the symbiotic treatment and the placebo groups.

4 | DISCUSSION

Irrespective of the causal factor, literature has indicated that RAS occurs through epithelial destruction mediated by a cellular immune response. Based on this principle, the present study sought to influence this destructive phenomenon, using a strategy to stimulate regulatory immune response through a symbiotic-based treatment, independent of the primary causes, which in most cases cannot be identified.

TABLE 4 Changing of the immune response after 6 mo of follow-up (n = 45)

Immune profile/ time point	Groups	Number of cases			OR (IC95)	*P*
		Decreased (1→0)	Maintained (0→0)	Increased (0→1)		
Th0						
T4-T0	Placebo	10 (43.5%)	12 (52.2%)	1 (4.3%)	1	*<.05*
	Symbiotic	3 (13.6%)	15 (68.2%)	4 (18.2%)	4.9 (1.3-18.3)	
T6-T0	Placebo	9 (39.1%)	9 (39.1%)	5 (21.7%)	1	*.143*
	Symbiotic	3 (13.6%)	13 (59.1%)	6 (27.3%)	2.3 (0.7-7.2)	
Th1						
T4-T0	Placebo	1 (4.3%)	21 (91.3%)	1 (4.3.%)	1 (0.0-17.0)	*1.000*
	Symbiotic	0 (0.0%)	22 (100.0%)	0 (0.0%)	1	
T6-T0	Placebo	0 (0.0%)	22 (95.7%)	1 (4.3%)	1	*.532*
	Symbiotic	0 (0.0%)	20 (90.9%)	2 (9.1%)	2.2 (0.2-26.1)	
Th2						
T4-T0	Placebo	1 (4.3%)	11 (47.8%)	11 (47.8%)	4.6 (1.3-16.0)	*<.05*
	Symbiotic	5 (22.7%)	13 (59.1%)	4 (18.2%)	1	
T6-T0	Placebo	4 (17.4%)	10 (43.5%)	9 (39.1%)	3.6 (1.1-11.8)	*<.05*
	Symbiotic	7 (31.8%)	13 (59.1%)	2 (9.1%)	1	
Mix						
T4-T0	Placebo	2 (8.7%)	20 (87.0%)	1 (4.3%)	1	*.716*
	Symbiotic	2 (9.1%)	18 (81.8%)	2 (9.1%)	1.35 (0.3-6.8)	
T6-T0	Placebo	2 (8.7%)	21 (91.3%)	0 (0.0%)	1	*.441*
	Symbiotic	2 (9.1%)	18 (81.8%)	2 (9.1%)	2.0 (0.3-12.3)	

The table shows the distribution of cases before and after the treatment between 2 time points (T4 and T6). The cases named as Decreased correspond to those had an immune profile in T0 classified as 1 (outside of the normal range of cytokine level) and lost this classification in T4 or T6. The cases marked as Increase changed its classification from 0 (outside of the normal range of cytokine level) into 1 (inside of the normal range) between time points. The cases signed as Maintained did not suffer alteration in the immune profile between 2 intervals. The data showed that between the time points T4 and T0, the treatment increased significantly the odds of symbiotic in change the immune profile to Th0 subtype. On the other side, the lack of intervention increased significantly the odds of placebo in change the immune profile to Th2 subtype between the both intervals: T4-T0 and T6-T0. OR: odds ratio; CI 95: 0.95 confidence interval.

[a]Ordinal logistic regression test.

To achieve this, it was of fundamental importance to define the initial serological immune status of the patients at T0 and to monitor how this state changed during treatment. We recorded significant differences between the levels of IFN-γ (Th1), IL-4 (Th2), and IL-6 (inflammatory) cytokines of the RAS patients in comparison with control (Figure 1). These data agree with the majority of published studies which show a tendency for increased Th1 response in RAS patients.[4,10]However, the few studies that have analyzed the Th1/Th2 serological status of RAS patients have obtained inconsistent results: Th1,[11] Th2,[12] or mixed.[13] In contrast to other studies that examined the collective Th1/Th2 serum cytokine profile, we compared the immune status of the patients individually and thus were able to divide the RAS group into 2 contingents, one of which had high levels of Th2 and the other which had increased levels of both Th1 and Th2 serum cytokines (mixed). Possibly, there are groups of affected individuals that possess distinct alterations that lead to the common dysfunctions in pathways responsible for the onset of RAS.

Nevertheless, despite the Th1 nature of RAS, a set of patients has been found to possess a history of atopy and a high level of serological IgE and IL-4, which are related to Th2 immune response.[14,15] The fact that aphthae lesions are associated with a Th1 immune response does not exclude the possibility that the patient possesses a concomitant hyperreactive Th2 disorder, such as occurs in Behcet's disease.[16]

On the other hand, the possibility of overlapping inflammatory diseases with different profiles cannot be ruled out in the present study. In such cases, it is probable that the lack of activity in the regulatory pathways that modulate both types of immune responses induces an intense Th1 and Th2 immune response in different mucous, with a distinct predilection for a type of T lymphocyte (such as buccal, nasal, and pulmonary).[17] The blood, draining the cytokines liberated by the mucus, could then exhibit the combined profile produced by different tissues.

Reinforcing the above hypothesis, our data showed that the level of IL-4 increased in T4 and decreased in T6, irrespective of the

TABLE 5 Results of the clinical evaluation during the 6 mo of follow-up

Clinical characteristic	Time (mo)	Symbiotic (mean ± SD)	Placebo (mean ± SD)	*P* treatment	*P* time
Total number of lesions	0	7.8 ± 7.0[a]	7.3 ± 4.5[a]	.864	.000*
	4	4.3 ± 4.7[b]	3.6 ± 3.6[b]		
	6	1.9 ± 1.8[b]	2.6 ± 2.1[b]		
PAIN (according VAS)	0	6.2 ± 1.1[a]	5.9 ± 1.9[a]	.006*	.000*
	4	2.6 ± 2.1[b]	3.6 ± 3.2[b]		
	6	1.6 ± 1.5[b]	3.3 ± 2.7[b]		
Average healing time (d)	0	10.8 ± 5.9[a]	9.8 ± 3.9[a]	.256	.000*
	4	5.0 ± 3.1[b]	6.0 ± 5.5[b]		
	6	4.4 ± 3.6[b]	5.2 ± 3.4[b]		
Maximum lesion size (millimeters)	0	5.7 ± 3.4[a]	5.6 ± 1.6[a]	.155	.000*
	4	3.6 ± 2.7[b]	3.7 ± 2.9[b]		
	6	2.7 ± 2.1[b]	3.7 ± 2.4[b]		
Number of outbreaks	0	2.7 ± 1.7[a]	3.0 ± 1.5[a]	.430	.000*
	4	2.0 ± 1.5[a]	1.8 ± 1.6[b]		
	6	1.4 ± 1.6[b]	1.6 ± 1.6[b]		

TNL: total number of lesions; AHT: average healing time; MLS: maximum lesion size; NO: number of outbreaks.

P treatment analyzed by Mann-Whitney U text-Fisher's exact test. *P* time analyzed by Kruskal-Wallis test.

Distinct letters (superscript) for mean values indicate significant differences between the 3 different moments of the study (baseline, 4 mo, and 6 mo) according to post hoc pairwise comparisons analyzed by Dunn test adjusted by Benjamini-Hochberg methodology.

*Results were considered significant when a *P*-value was <.05.

group (Table 3). The increase in IL-4 was related to the random conjunction of 2 factors: seasonality and Th2 immune profile of the participants. A great number of patients with Th2 profile (n = 8 from 11) was scheduled for blood withdraw (T4) in the fall season. The incidence of Th2 respiratory diseases, like asthma, is particularly higher in large cities in this season. As this research was conducted in São Paulo, the Brazil's largest city, it can be speculated that the IL-4 increase was caused by a higher incidence of respiratory diseases in the Th2 patients in T4 time point (Figure 1). However, as the selection of the patients was random, the phenomenon affected both groups (symbiotic and placebo) in the same way.

Recently, the literature has reinforced the relationship between RAS and hematinic deficiencies. However, the nature of this relationship is uncertain, as vitamin B12 treatment improves the disease regardless of the initial vitamin level.[18] On the other hand, the presence of autoantibodies (Abs) against gastrointestinal cells seems to play a significant role in causing vitamin B12 deficiency in RAS.[19] It cannot be determined whether these factors are linked to the origin of the disease or are associated with the same altered immune condition. Although the presence of autoantibodies was often related to the exaggerated Th2 immune response, the present study did not evaluate the hematological status of the patients, and so we cannot rule out the possibility that our patients with Th2 or Th1/Th2 polarization possess concomitant hematological deficiency.

Probiotics modulate the production of cytokines, and this modulation depends on the strain used. Dong et al (2012) compared the effects of 6 probiotic strains on in vitro immune responses and observed that all *Lactobacillus* strains induced the production of Th1 cytokines, while bifidobacteria tended to induce the secretion of anti-inflammatory cytokines.[20] The symbiotic composition tested in the present study presented both *Lactobacillus* and bifidobacteria, which may explain the responses observed in patients, who exhibited increased IFN-γ levels, mainly at T6. The improved levels of IFN-γ were probably associated with the relative increase in the number of patients with Th0 profiles and with the decrease of the number of patients classified as Th2 in the group receiving symbiotic treatment (Table 4).

Regarding the clinical results, the only parameter that improved in the present study was the pain. In study of Trinchieri et al[21], tablets containing the probiotic *Lactobacillus brevis* CD2 or placebo were given to the RAS patients for a period of 7 days and the levels of prostaglandin, IFN-γ, nitrite, nitrate, and nitrous oxide from saliva samples were evaluated. The results showed a decrease in the laboratorial and clinical parameters of the RAS patients. The literature has demonstrated that probiotics are capable of controlling pain by modulating the activities of genes involved in nociception such as tryptophan hydroxylase 1 (TPH-1), cannabinoid receptor 1 (CNR1), and opiate receptor-like 1.[22] Clinical trials have showed that treatment with probiotic decrease the dolorous symptomatology in elderly patients with distal radius fracture [23] and in patients with abdominal pain.[24]

Despite having RAS patients with elevated levels of serum IL-4, the increase in INF-γ induced by this symbiotic composition may declassify the use of this compound for the treatment of Th1 immune type diseases such as RAS. Probiotics that work to reduce

Th1 response would probably have more success in controlling the disease. This contradiction could be explained by the fact that IL-4 was not directly associated with the onset of the RAS but was probably linked to a manifestation of an immune response of pulmonary origin.

The purpose of the present study was to support the well-being of RAS patients in a manner that improves their quality of life and does not simply reduce their symptoms. This is a preliminary and unprecedented study that provides new perspectives that merit further evaluation, especially using symbiotic or prebiotic formulas containing other combinations of microorganisms.

5 | CONCLUSIONS

In RAS, symbiotic treatment based on a fructooligosaccharide, Lactobacillus, and Bifidobacterium composition altered the Th2 serological immune profile in the direction of Th1 with the improvement of pain symptomatology.

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CONFLICT OF INTEREST

The authors confirming the absence of any conflict of interest.

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