

Cheese supplemented with probiotics reduced the *Candida* levels in denture wearers—RCT

TY Miyazima¹ | KH Ishikawa^{1,2}  | MPA Mayer² | SMI Saad³ | AEM Nakamae¹

¹Department of Prosthodontics, School of Dentistry, University of São Paulo, São Paulo, SP, Brazil

²Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

³Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil

Correspondence

Atlas Edson Moleros Nakamae, Department of Prosthodontics, School of Dentistry, University of São Paulo, São Paulo, SP, Brazil. Email: atlas@usp.br

Funding information

Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2010/01323-0; National Council of Scientific and Technological Development, Grant/Award Number: 140.1462012-1

Objectives: The access to probiotics should be facilitated in order to encourage their usage. We evaluated the effect of consumption of two experimental probiotic-containing cheeses on the oral colonization of *Candida* in denture wearers.

Methods: Sixty denture wearers harboring oral *Candida* were randomly allocated in groups who received cheese supplemented with *Lactobacillus acidophilus* NCFM (T1) or *Lactobacillus rhamnosus* Lr-32 (T2), daily for 8 weeks, and a control group (C) who received a control cheese. Oral samples were obtained through a mouthwash, and *Candida* levels were determined (CFU/mL) at baseline and after the 8-week experimental period.

Results: At baseline, the mean levels of *Candida* spp. (log CFU/mL) were similar among the groups. However, the mean levels of *Candida* were significantly reduced in groups T1 and T2 but not in C (Tukey, $p < .05$). The reduction in *Candida* oral levels occurred independently on the colonizing *Candida* species, participant age, and use of bi- or uni-maxillary dentures.

Conclusions: Daily consumption of cheese supplemented with probiotics, with either *L. acidophilus* NCFM or *L. rhamnosus* Lr-32, was able to reduce the colonization of oral *Candida* in complete denture wearers, suggesting its potential in reducing the risk of oral candidiasis in this highly susceptible population.

KEYWORDS

Candida, complete denture, edentulous, *Lactobacillus*, probiotics

1 | INTRODUCTION

Species of the genus *Candida* are resident in the oral cavity and gut, and enteric colonization by *Candida* is considered the most important risk factor for invasive candidiasis (Kumar & Singhi, 2013). Predisposing factors may break the balance between the resident microbiota and the host, leading to diseases associated with *Candida* overgrowth ranging from superficial infections to severe systemic and pervasive disseminations (Ghannoum et al., 2010; Samaranyake, Keung Leung & Jin, 2009).

Complete denture wearers are more likely to develop oral candidiasis (Reichart, 2000) and present higher rates of prevalence of *Candida* spp. (Lyon, da Costa, Totti, Munhoz & de Resende, 2006)

when compared with those who do not wear complete denture. Furthermore, elderly are more susceptible to *Candida* infection due to systemic conditions such as diabetes, xerostomia, immune deficiencies, and local factors such as ill-adapted dentures and poor oral and prosthesis hygiene, which facilitate *Candida* colonization (Salerno et al., 2011). Thus, methods to reduce the oral colonization by *Candida* in candidiasis-prone subjects are needed. These methods should not suppress the resident microbiota but rather provide the maintenance of host homeostasis by maintaining a microbiota associated with health.

Probiotic bacteria are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). These benefits may include the inhibition of potentially

pathogenic microorganisms, by the production of antimicrobial substances (Burton, Chilcott, Moore, Speiser & Tagg, 2006; Sookkhee, Chulasiri & Prachyabrued, 2001) and modulation of local and systemic immune system (Plantinga et al., 2012; Villena, Salva, Agüero & Alvarez, 2011). Previous data have shown that the usage of probiotics may prevent or treat candidiasis (Kumar & Singhi, 2013; Ronnqvist, Forsgren-Brusk, Husmark & Grahn-Hakansson, 2007). In vitro (Hasslof, Hedberg, Twetman & Stecksen-Blicks, 2010; Strus et al., 2005) and in vivo studies (Elahi, Pang, Ashman & Clancy, 2005; Hatakka et al., 2007; Matsubara, Silva, Paula, Ishikawa & Nakamae, 2012) demonstrated the inhibitory effect of probiotics over *Candida*. Furthermore, our group has recently reported that the usage of probiotic-containing capsules was able to reduce *Candida* colonization levels in denture wearers (Ishikawa et al., 2015).

Probiotics are usually administered as pharmaceutical products in tablets (Mayanagi et al., 2009) or lozenges (Caglar, Kuscu, Cildir, Kuvvetli & Sandalli, 2008), but they can also be added to chewing gums (Twetman et al., 2009), cheese (Hatakka et al., 2007), and fermented milk (Gill, Rutherford, Cross & Gopal, 2001). As these bacteria do not usually colonize the host permanently (Busscher, Mulder & van der Mei, 1999; Caglar, Topcuoglu, Cildir, Sandalli & Kulekci, 2009; Meurman & Stamatova, 2007; Yli-Knuutila, Snall, Kari & Meurman, 2006), their effect is dependent on the daily use over a long period. Thus, the addition of probiotics to foods may be a strategy to increase acceptability, as the beneficial microbial culture is daily delivered to the target population without introducing changes in the patient's routine.

This study aimed to investigate the effect of the consumption of a Brazilian typical fresh white cheese supplemented with two probiotic lactobacilli strains (*L. acidophilus* NCFM and *L. rhamnosus* Lr-32) on the oral colonization of *Candida* spp. in denture wearers.

2 | MATERIALS AND METHODS

2.1 | Subjects and study design

The protocol was approved by the School of Dentistry Ethics Committee at the University of São Paulo (FR 331277, protocol 55/10). Written informed consent was obtained from all subjects. Clinical trial was registered in <http://www.ensaiosclinicos.gov.br/> (registration # RBR-5hn7gn). This was a randomized placebo-controlled, parallel, double-blind study, with three arms, conducted for 8 weeks. Subjects were randomly allocated in two experimental groups (T1—receiving cheese supplemented with *L. acidophilus* NCFM and T2—receiving cheese supplemented with *L. rhamnosus* Lr-32) and one control group (C—receiving cheese with no added probiotic strains). At baseline, demographic characteristics and clinical data (oral and general health) of each group were recorded.

Initially, 135 denture-wearing patients seeking for dental treatment (complete denture) at the School of Dentistry, University of São Paulo, Brazil, were selected. They were submitted to anamneses; clinical inspections of the palate, and the saliva were collected. According to the inclusion criteria, 66 subjects were excluded, and 69 were selected to participate of this study.

Inclusion criteria were oral colonization by *Candida* and absence of clinical signs of denture stomatitis or candidiasis. Hypertension, cardiac diseases, and diabetes are considering problems very common in the most susceptible population, and then, they were included in this study. Patients reporting lactose or milk-based food intolerance, kidney problems, head and neck cancer, or radiotherapy after this disease, those not able to follow the complete instructions, and those who had received topic or systemic antifungal or antibacterial agents in the previous 60 days were excluded. Nine subjects were excluded due to refusal to consume cheese daily. The selected sixty subjects were randomly allocated to one of the three groups, 20 subjects in each group. At baseline, all participants received oral and denture hygiene instructions and were asked not to use oral rinse products and other probiotic-containing products during the experimental period.

2.2 | Sample collection

Oral samples were obtained at baseline and immediately after the 4- and 8-week experimental period. All participants were instructed to avoid eating, drinking (except water), and smoking, 1 hr before sample collection. The participants were asked to rinse their mouths with 10 mL of sterile saline for 60 s (Samaranayake, Macfarlane, Lamey & Ferguson, 1986). The mouth-rinse samples were then dispensed in plastic vials and immediately transferred to the laboratory.

2.3 | Microbiological analysis

Mouth-rinse samples were centrifuged at 1,700 g for 10 min, and the sediments were resuspended in 3 mL of sterile saline. Aliquots of decimal dilutions were inoculated in triplicate onto the surface of Sabouraud dextrose agar plates (Difco Laboratories, Detroit, MI, USA) with chloramphenicol (0.05 g/L), and incubated for 24 hr at 37°C. After growth, colonies were counted and the number of CFU *Candida*/mL of mouth rinse was determined. The diluted samples were also inoculated on the surface of CHROMagar *Candida*® (CHROMagar, Paris, France) and incubated aerobically at 37°C for 72 hr. After this period, the *Candida* species were presumptively identified by colony morphology and submitted to specie identification by biochemical tests (API 20C® Aux assimilation test, BioMérieux, Basingstoke, UK), germ tube and chlamyospore formation and sugar fermentation. *C. albicans* and *C. dubliniensis* were differentiated by growth at 42–45°C and in hypertonic medium (Sabouraud dextrose broth added with 6.5% of NaCl), as only *C. albicans* can withstand these growth conditions.

2.4 | Fresh white cheese manufacture

Probiotics comprised freeze-dried cells of *L. acidophilus* NCFM and *L. rhamnosus* Lr-32 (DuPont™ Danisco®, São Paulo, Brazil) provided by the manufacturer.

Fresh cheese (Brazilian Minas fresh white cheese) was produced as recommended (Buriti, Rocha & Saad, 2005; Souza & Saad, 2009). Lactic acid (0.25 mL L⁻¹) was added to 10 L vats of commercial pasteurized milk (Xandô, São Paulo, Brazil) followed by heating

to 36–37°C. Then, 0.25 g/L of *L. acidophilus* NCFM or 0.35 g/L of *L. rhamnosus* Lr-32 was added to the experimental cheese in order to achieve 7 to 8 log CFU g⁻¹, followed by the addition of commercial rennet (85% bovine pepsin + 15% bovine chymosin; Estrela, Christian Hansen, Valinhos, Brazil, 50 mg/L) and calcium chloride (2.5 g/L) to the cheese milk. All vats were then allowed to set at 36°C, until a firm curd was formed (ca. 50 min), which was gently diced, transferred to a vessel, and allowed to drain. Two liters of whey was removed and 40 g of salt (NaCl) was added. After overnight draining, the cheeses were placed in sealed plastic bags and stored under refrigeration (5°C) for up to 21 days.

The cheese was proved to be safe for consumption, with strict quality control in production and storage, ensuring the absence of contaminants and the appropriate viability of probiotic bacteria until final consumption. The cheeses supplemented with probiotics contained 8 to 9 log CFU g⁻¹ of *L. acidophilus* NCFM or *L. rhamnosus* Lr-32, whereas the control cheese had no added probiotics. All cheese had similar flavor, texture, and color. Probiotics viability was determined in each cheese batch at days 1, 7, and 14.

2.5 | Intervention

All participants received packages, containing individual portions of 20 g of fresh white cheese, every 2 weeks for eight consecutive weeks. These packages were identified with A, B, or C, according to the subject's group allocation. The experimental groups received the cheese supplemented with probiotics *L. acidophilus* NCFM (T1) or *L. rhamnosus* Lr-32 (T2), whereas the control group (C) received the cheese without probiotic supplementation. The participants were interviewed about their cheese consumption and asked to report any intercurrent, and given a new batch of cheese, every 2 weeks during the experiment period.

2.6 | Statistical analysis

Sample size calculation was based in our previous data (Ishikawa et al., 2015), which indicated a reduction in *Candida* levels in 83% subjects after probiotics usage. A total sample of 66 subjects was considered to be adequate to achieve 80% power, $p < .05$, 95% confidence interval. However, from 69 subjects selected to participate of this study, nine patients were excluded after baseline data were collected due to refusal to each cheese, and the study comprised 60 subjects.

Kolmogorov–Smirnov test was used to determinate normal distribution of almost all variables studied. As not all quantitative variables had this distribution, we chose nonparametric tests. Chi-square (X^2) was used to test for differences in qualitative (gender, ethnical background, self-reported conditions and use of uni- or bimaxillary prosthesis) and quantitative variables (*Candida* levels, patient age, and age of the dentures) among the groups (T1, T2, and C) at baseline. Fisher test was used to access for differences in the number of highly infected subjects at baseline and after the experimental period. Tukey's multiple comparisons test was used to compare the mean levels of *Candida* spp. in the oral samples among the groups, and between baseline,

the 4- and 8-week experimental period. Sidak's multiple comparisons test as used to verify differences in the prevalence of *Candida* species among the groups at both studied periods. Statistical significances were considered at $p \leq .05$.

Statistical analyses were performed with Statistics software to Windows version 15.0 (SPSS Inc., Chicago, USA) and GraphPad Prism® (version 6.0c—GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

Sixty-nine denture wearers harboring *Candida* in the oral cavity with no clinical symptoms were selected for participation in this double-blind randomized clinical study. During the study, nine refused to eat cheese. Thus, demographic and clinical characteristics of the 60 subjects allocated in three studied groups who completed the experimental period are shown in Table 1. There were no significant differences among the three groups regarding the variables evaluated at baseline ($p > .05$, X^2). However, it should be noticed that 95% of the subjects allocated in the control group presented only maxillary dentures, whereas a higher prevalence of bimaxillary dentures was shown in the experimental groups. One subject of group T1 was excluded from the study during the experimental period due to refusal to continue eating cheese during the 8-week experimental period. The number of 10^{7–8}CFU probiotic/sample was maintained throughout the study, in different batches of cheese.

3.1 | *Candida* spp. levels at baseline and after the experimental period

Although all subjects (60 subjects, 20 of each group) had detectable levels of *Candida* spp, at baseline and at the end of the experimental period, this genus was not detected in two patients of each experimental group and in three control subjects. The mean levels of *Candida* at baseline and after 4 and 8 weeks of the experimental period are shown in Figure 1. A reduction in *Candida* levels was observed for the three studied groups. This reduction was continuous throughout the experimental period, and after 4 weeks of consumption of cheese with probiotic, there was a significant reduction in *Candida* levels only for T1 (*L. acidophilus* NCFM). However, when baseline *Candida* levels were compared with data obtained after the 8-week period, a significant difference in *Candida* oral levels was observed for both experimental groups, and not for the control group.

Furthermore, the reduction in the number of highly infected subjects (≥ 3 log CFU mL⁻¹) was more pronounced in both experimental groups than in the control group (Table 2). The number of highly infected subjects was higher in T1 than in T2 and C groups, although this difference was not significant. Thus, the reduction in the number of *Candida* highly infected subjects was significant only for the T1 group ($p < .05$, Fisher test), but not for the other groups.



Qualitative variables	Category	Groups			p (χ^2)
		T1 ^a N° (%)	T2 ^b N° (%)	Control N° (%)	
Ethnicity	Caucasian	14 (73.7)	10 (50.0)	12 (60.0)	.315
	Afro-descendant	5 (26.3)	10 (50.0)	8 (40.0)	
Gender	Female	15 (78.9)	17 (85.0)	17 (85.0)	.846
	Male	4 (21.1)	3 (15.0)	3 (15.0)	
Mucosal lesion	No	15 (78.9)	15 (75.0)	15 (75.0)	.946
	Yes	4 (21.1)	5 (25.0)	5 (25.0)	
Type of complete denture	Maxillary	13 (68.4)	13 (65.0)	19 (95.0)	.052
	Mandibular	6 (31.6)	7 (35.0)	1 (5.0)	
Diabetes	No	13 (68.4)	15 (75.0)	17 (85.0)	.471
	Yes	6 (31.6)	5 (25.0)	3 (15.0)	
Hypertension	No	8 (42.1)	9 (45.0)	8 (40.0)	.950
	Yes	11 (57.9)	11 (55.0)	12 (60.0)	
Cardiac diseases	No	18 (94.7)	18 (90.0)	19 (95.0)	0.780
	Yes	1 (5.3)	2 (10.0)	1 (5.0)	
Quantitative variables		Mean \pm SD			p (χ^2)
Age (years)		66.1 \pm 11.6	61.7 \pm 14.1	65.5 \pm 10.5	.166
Upper jaw denture age (years)		15.7 \pm 12.9	12.7 \pm 11.2	12.7 \pm 9.4	.740
Lower jaw denture age (years)		9.6 \pm 9.6	11.2 \pm 10.7	10.5 \pm 7.0	.751
<i>Candida</i> infection level (log ₁₀ CFU/mL)		3.5 \pm 0.41	3.2 \pm 0.51	3.3 \pm 0.49	.166
Total		19	20	20	

^aT1 = cheese with *L. acidophilus* NCFM.

^bT2 = cheese with *L. rhamnosus* Lr-32 (Danisco, Dange, France).

3.2 | Distribution and prevalence of *Candida* species

C. albicans was the most prevalent yeast isolated from the participants at baseline, that is, 67.8% of patients harbored only *C. albicans*, 30.5% harbored non-*albicans*, and 1.7% presented both *C. albicans* and non-*albicans* species. Among non-*albicans* *Candida* species, *C. glabrata* was the most frequently detected (16.9%), followed by *C. dubliniensis* (6.8%), *C. tropicalis* (5.1%), *C. krusei* and *C. famata* (3.4% each), and *C. kefyr* (1.6%).

After the experimental period, *C. albicans* was present in 57.6% of the patients, non-*albicans* was present in 28.8%, and *C. albicans* associated with non-*albicans* species was present in 1.7%. *C. glabrata* remained the second most frequently detected yeast (15.3%), followed by *C. dubliniensis* (3.4%), *C. tropicalis* (11.9%), and *C. kefyr* (1.7%). *C. krusei* and *C. famata* were not detected at the final samples. There were no differences in the prevalence of *Candida* species among the three groups after cheese intake ($p > .05$, Sidak's multiple comparisons test).

The colonizing *Candida* specie (*C. albicans* or non-*albicans*) and other qualitative (Table 1) or quantitative variables, such as self-reported diabetes, patient's and denture's age, exerted no influence on the reduction of *Candida* levels promoted by probiotics ($p > .05$, χ^2).

TABLE 1 Distribution of qualitative and quantitative variables (demographic, self-reported, and clinical features) of patients according to the groups studied: Groups T1 and T2 received the probiotic-containing cheese, whereas the control group received cheese without the addition of probiotics

4 | DISCUSSION

The use of probiotics in food is an emerging field, which can contribute to the maintenance of health in humans (Stanton, Ross, Fitzgerald & Van Sinderen, 2005; Vanderhoof et al., 1999).

In the present study, we have shown that the consumption of cheese containing probiotic bacteria is able to reduce the oral colonization of species of *Candida* in a population who is highly susceptible to oral candidiasis. This reduction was achieved in elderly subjects, presenting aged dentures and even conditions such as diabetes.

The benefits provided by probiotics are strain specific, and thus, their effect may be tested for each strain (Guarner & Malagelada, 2003). In the present study, both tested probiotic strains were able to reduce *Candida* spp. infection levels. Previous data indicated that the use of a mixture of several probiotic bacteria (*Lactococcus lactis*, *L. helveticus*, *L. rhamnosus* GG, *L. rhamnosus* LC705, and *Propionibacterium freudereichi*ssp. *sermanii* JS) in emmental cheese was able to reduce the risk of high *Candida* counts by 75% (Hatakka et al., 2007), but there was no report on the effect of each strain. Our previous data (Ishikawa et al., 2015) demonstrated that the addition of a capsule containing lyophilized *L. rhamnosus* HS111 and *L. acidophilus* H101 to the dentures led to a reduction in *Candida*

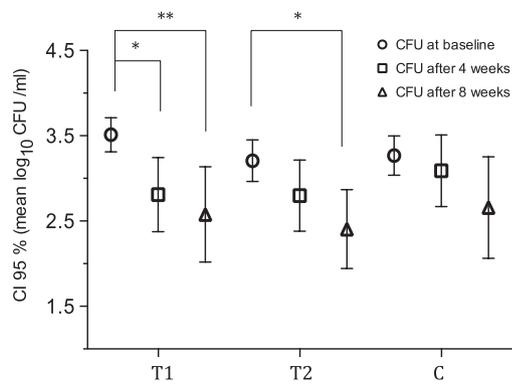


FIGURE 1 *Candida* levels at baseline and after a 4- and 8-week experimental period, according to groups: T1 (cheese with *L. acidophilus* NCFM), T2 (cheese with *L. rhamnosus* Lr-32), and control (cheese without probiotics). **Significant difference in *Candida* levels (Tukey, $p < .01$). *Significant difference in *Candida* levels (Tukey, $p < .05$)

colonization levels but whether this effect was due to one or both strains was not elucidated.

The strains tested in the present study have also been previously evaluated in experimentally infected DBA/2 immunosuppressed mice, and both probiotic strains, *L. acidophilus* NCFM and *L. rhamnosus* Lr-32, were able to reduce the oral colonization of *C. albicans*. However, in the mice model, treatment with *L. rhamnosus* Lr-32 was more effective than the treatment with *L. acidophilus* NCFM and even than the positive control treatment with nystatin (Matsubara et al., 2012). The probiotic treatment tended to decrease also the number of highly infected subjects. However, our data indicating that only the treatment with *L. acidophilus*, and not with *L. rhamnosus*, promoted a reduction in the number of *Candida* highly infected subjects ($\log \text{CFU/mL} \geq 3$) should be taken with caution, since the number of subjects with high oral *Candida* levels among groups were different at baseline (Table 2). Furthermore, these data should be confirmed in a larger population, in a longer evaluation period, aiming to elucidate whether the reduction in *Candida* levels promoted by the probiotics added to cheese result in decreased frequency of candidiasis events.

In the present study, a significant reduction in oral *Candida* spp. levels was observed for the experimental groups between baseline and 8-week samples ($p < .05$), but not for the control group. However, a non-significant reduction was also seen for the control group, suggesting that part of the decrease observed in the experimental groups may have been promoted by factors such as improvement in oral and denture hygiene due to the instructions given to all participants at baseline.

Data on systemic diseases and medical history were obtained from all patients at baseline. No patients reporting radiotherapy after head and neck cancer were selected. Furthermore, there were no differences (χ^2 , $p > .05$) on the prevalence of hypertension, diabetes, and cardiac diseases at baseline among the groups (Table 1). Furthermore, these variables imposed no effect on the reduction in *Candida* levels promoted by probiotics (χ^2 , $p > .05$). Although diabetes and patients with hypertension/cardiac diseases present decreased salivary flow in relation to healthy subjects, these conditions are very common among elderly, denture wearer subjects, thus increasing their susceptibility to oral candidiasis. Therefore, we consider that these more prone patients should be included in any study to test the effect of probiotics. Furthermore, a decreased salivary flow may even improve the effects of probiotics, by increasing their clearance time from the oral cavity.

The significant but rather low reduction in *Candida* infection levels observed in this study promoted by the probiotics may be due to the low frequency of usage (1x/day), number of probiotic cells, and delivery system (cheese), which will exert an effect on the period of probiotics maintenance at the oral cavity. These observations are reinforced by our previous report, in which the application of probiotics directly to the palatal surface of dentures led to a higher reduction in *Candida* spp. levels (Ishikawa et al., 2015).

Although the loading of probiotics in cheese is a viable way, the period of the cheese stay in the oral cavity is only during the masticatory cycle, and the probiotics is not in direct contact to the palatal surface, due to the upper complete denture that covers the palate. Thus, factors that increased probiotic clearance, such as increased salivary flow rate due to chewing and cheese tasting, may have negatively influenced the reduction in *Candida* in the present study. In contrast,

TABLE 2 Distribution of subjects allocated in groups (T1, T2, and C), according to *Candida* spp. infection levels at baseline and after the 8-week experimental period

<i>Candida</i> infection level (log CFU/mL)	At baseline			After the 8-week period		
	T 1 N (%)	T 2 N (%)	C N (%)	T 1 N (%)	T 2 N (%)	C N (%)
Non detected	0	0	0	2 (10.5)	2 (10.0)	3 (15.0)
$1 \leq x < 3$	2 (10.5)	6 (30.0)	6 (30.0)	9 (47.4)	10 (50.0)	5 (25.0)
$x \geq 3$	17 (89.5)	14 (70.0)	14 (70.0)	8 (42.1) ^a	8 (40.0)	12 (60.0)
Total	19 (100.0)	20 (100.0)	20 (100.0)	19 (100.0)	20 (100.0)	20 (100.0)
	$p (\chi^2) = 0.259^b$			$p (\chi^2) = 0.3817^c$		

T1: received cheese supplemented with *L. acidophilus* NCFM.

T2 received cheese supplemented with *L. rhamnosus* Lr-32 (T2).

C: cheese without probiotics.

^aSignificant reductions in *Candida* infection levels between baseline and 8-week ($p < .05$, Fisher test).

the addition of the probiotic strains to the palatal surface of a denture may maintain the probiotic cells for a longer period in contact with the mucosa surface in a saliva flow-limited environment. Therefore, an increased effect on *Candida* levels by probiotics should possibly be obtained if higher concentrations of beneficial bacteria in a more viscous vehicle than fresh cheese were used.

A significant finding in our study is that factors facilitating the *Candida* oral colonization such as diabetes, use of bimaxillary dentures, and age did not affect the reduction in *Candida* levels of the experimental groups. Furthermore, the species of colonizing *Candida* did not seem to influence the effect of the probiotics. This is particularly relevant in *C. glabrata* infections, as this species is usually more resistant to antifungal drugs than *C. albicans*.

5 | CONCLUSIONS

Daily consumption of cheese supplemented with probiotics, with either *L. acidophilus* NCFM or *L. rhamnosus* Lr-32, was able to reduce the colonization of oral *Candida* in complete denture wearers, suggesting their potential in reducing the risk of oral candidiasis in these highly susceptible subjects.

ACKNOWLEDGEMENTS

The study was financially supported by the National Council of Scientific and Technological Development (CNPq, Brazil grant# 140.1462012-1) and Fundação de Amparo a Pesquisa de São Paulo (FAPESP grant # 2010/01323-0). We would also like to thank Companies Xandô for donating the milk for the production of the cheese and DuPont™Danisco® for donating the probiotic strains. The authors declare no conflict of interests in this study.

AUTHOR CONTRIBUTIONS

Miyazima – conducted research, designed study and drafted paper
Ishikawa – conducted research, designed study, analyzed data and drafted paper
Mayer – analyzed data and drafted paper
Saad – designed study of cheese manufacturing
Nakamae – conducted research and drafted paper.

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How to cite this article: Miyazima TY, Ishikawa KH, Mayer MPA, Saad SMI, Nakamae AEM. Cheese supplemented with probiotics reduced the *Candida* levels in denture wearers—RCT. *Oral Dis*. 2017;23:919–925. <https://doi.org/10.1111/odi.12669>