ACTA ODONTOLOGICA SCANDINAVICA
2019, VOL. 77, NO. 7, 517–524
https://doi.org/10.1080/00016357.2019.1609697

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Effect of ingesting yogurt fermented with *Lactobacillus delbrueckii ssp. bulgaricus* OLL1073R-1 on influenza virus-bound salivary IgA in elderly residents of nursing homes: a randomized controlled trial

Yuko Yamamoto[a], Juri Saruta[b], Toru Takahashi[c], Masahiro To[d], Tomoko Shimizu[e], Takashi Hayashi[b], Toshiya Morozumi[f], Nobuhisa Kubota[b], Yohei Kamata[e], Seiya Makino[g], Hiroshi Kano[g], Jun Hemmi[g], Yukio Asami[g], Takayuki Nagai[h], Kyoko Misawa[i], Sachiko Kato[j] and Keiichi Tsukinoki[b]

[a]Department of Dental Hygiene, Kanagawa Dental University Junior College, Yokosuka, Japan; [b]Division of Environmental Pathology, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan; [c]Department of Food and Nutrition, Koriyama Women's University, Koriyama, Japan; [d]Division of Dental Anatomy, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan; [e]Department of Highly Advanced Stomatology, Graduate School of Dentistry, Kanagawa Dental University, Yokohama, Japan; [f]Division of Periodontology, Department of Oral Interdisciplinary Medicine, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Japan; [g]R&D Division, Food Microbiology Research Laboratories, Meiji Co. Ltd., Hachioji, Japan; [h]Department of Drug Discovery Sciences, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan; [i]Honobono, Elderly Nursing Home, Yokohama, Japan; [j]Elderly Nursing Home, Yokohama, Japan

ABSTRACT

Objective: The purpose of this study was to clarify the influence of consuming yogurt fermented with *Lactobacillus delbrueckii ssp. bulgaricus* OLL1073R-1 (1073R-1-yogurt) on influenza virus-bound salivary immunoglobulin A (IgA) levels, in the elderly residents of nursing homes.

Methods: A double-blind, parallel-group, randomized controlled trial was conducted with 96 elderly volunteers residing in 2 nursing homes. During the trial, participants consumed 100 g of 1073R-1-yogurt every morning for 12 weeks, whereas the control participants consumed yogurt fermented with a different *Lactobacillus* strain (control yogurt). Saliva was collected before the trial and after 4, 8 and 12 weeks of yogurt ingestion.

Results: Our data indicated that consumption of 1073R-1-yogurt affected influenza A virus subtype H3N2-bound IgA levels in saliva ($p = .001$). In addition, saliva flow rate and total IgA levels increased in response to the yogurt intake period in both the 1073R-1 and control yogurt groups ($p = .04$).

Conclusions: Our study suggests that continuous daily ingestion of 1073R-1-yogurt may help prevent infection with influenza A virus subtype H3N2 in elderly subjects with weakened immunity, by increasing the production of influenza A virus subtype of H3N2-bound salivary IgA.

ARTICLE HISTORY
Received 21 January 2019
Revised 24 March 2019
Accepted 10 April 2019

KEYWORDS
Saliva; IgA; yogurt; influenza; elderly population

Introduction

Saliva plays a vital role in both the local and systemic defense of the teeth, oral cavity, pharynx, larynx and upper digestive tract [1–3]. The normal saliva flow rate is also critical for the maintenance of oral and systemic health [4]. In addition, immunoglobulin A (IgA) is the most voluminous Ig found in the saliva, where it is critical to mucosal immunity [5–7]. Functionally, IgA mediates immune expulsion of harmful pathogens, prevents the growth of potentially harmful pathogens and limits the adhesion and permeation of antigens through the secretory epithelia. This reinforces the epithelial barrier, contributing to immunological homeostasis [8,9]. However, an age-related decrease in immune function is likely related to increased incidences of upper respiratory tract infections (URTI) and influenza virus infection [10,11]. In particular, seasonal influenza prevalence contributes seriously to worldwide disease load and deaths associated with lower respiratory infectious diseases in older people [12,13]. Seasonal influenza A (H3N2 and H1N1) and influenza B have been the three major epidemic virus subtypes in the world for many years [14]. Many seniors die every year owing to infection with these three subtypes of influenza virus [14]. Therefore, increasing levels of influenza virus-bound salivary IgA may represent an effective way to bolster the immune systems of the elderly population, potentially combating influenza virus infection.

Probiotics are defined as 'live microorganisms' that exert health benefits on the host when consumed in adequate amounts [15]. Among the different types of probiotics, many basic and clinical studies have been conducted specifically on the oral ingestion of *Lactobacillus* [16,17]. It has previously been reported that ingestion of *Lactobacillus ssp*. and yogurt fermented with *Lactobacillus ssp.* can regulate the immune response and also increase salivary IgA levels [18–22]. In particular, consumption of yogurt fermented with *Lactobacillus delbrueckii ssp. bulgaricus* (*L. bulgaricus*)

CONTACT Keiichi Tsukinoki tsukinoki@kdu.ac.jp Division of Environmental Pathology, Department of Oral Science, Kanagawa Dental University, Graduate School of Dentistry, 82 Inaoka, Yokosuka, Kanagawa 2388580, Japan

OLL1073R-1 (1073R-1-yogurt) was reported to enhance natural killer (NK) cell activity, decreasing the danger of older people infecting URTI [23]. A mouse model has also shown that 1073R-1-yogurt had anti-influenza virus immunopotentiating activity [24]. In this study, exopolysaccharides (EPSs) produced by *L. bulgaricus* OLL1073R-1 were found to be one of the most important underlying factors [24]. In our previous study, consumption of 1073R-1-yogurt led to increased total IgA levels in the saliva of elderly participants in a before-after, non-randomized intervention study [25]. However, the exact effects of 1073R-1-yogurt on influenza virus-bound salivary IgA are still unclear, and no double-blind test has yet been performed. The purpose of this study, therefore, was to clarify if the consumption of 1073R-1-yogurt by elderly people affected the saliva flow rate, the total amount of salivary IgA and the amount of influenza virus-bound salivary IgA in a multi-centre, double-blind and parallel-group randomized controlled trial.

Materials and methods

Participants

This study was a randomized, double-blind, parallel and placebo-controlled clinical trial conducted in several stages between October and December 2014. Elderly volunteers with limited activity and a mostly sedentary life were recruited from two nursing homes in Japan (Samukawa Home, Koza, Kanagawa and Honobono, Yokohama, Kanagawa). These two nursing homes were selected because of the similarity in their living environments, as both of them are general nursing homes situated in Japan. The following individuals were excluded from the study: individuals taking antibiotics or morphine, those with gastric fistulae, those who disliked yogurt, or those who were hospitalized and died during the study period. Initially, a total of 107 elderly volunteers consented to participate in this trial. These were randomly assigned to 2 groups, and each group was assigned meals (2–3 people per group) according to computer-generated random numbers. This assignment was managed by an independent professional not involved in this trial. Details regarding the allocation of the participants into two groups were only informed to the care staff at each nursing home. The care staff members were not involved in this study. Allocation details of the participants were not revealed to the researchers involved in the study until the end of data analysis. One group consumed 1073R-1-yogurt ($n = 54$), whereas the other had yogurt fermented with *L. bulgaricus* OLL1256 ($n = 53$). Ninety-six participants completed the entire course and were included in our analysis (a schematic overview is shown in Figure 1). Data on age, sex, stature, body weight, level of required care (graded between 1 and 5, based on long-term care insurance policies evaluated by a professional and based on an evaluation of care necessities) and dementia-level (the dementia status of the elderly population was evaluated at 7 levels, viz., I, IIa, IIb, IIIa, IIIb, IV and M. I represented mild dementia while M represented severe dementia [26], and was evaluated by an attending physician) were gathered from participants' nursing care records.

Ethical statement

The study was approved by the Ethics Committees of Kanagawa Dental University in 2014 (approval number: 287)

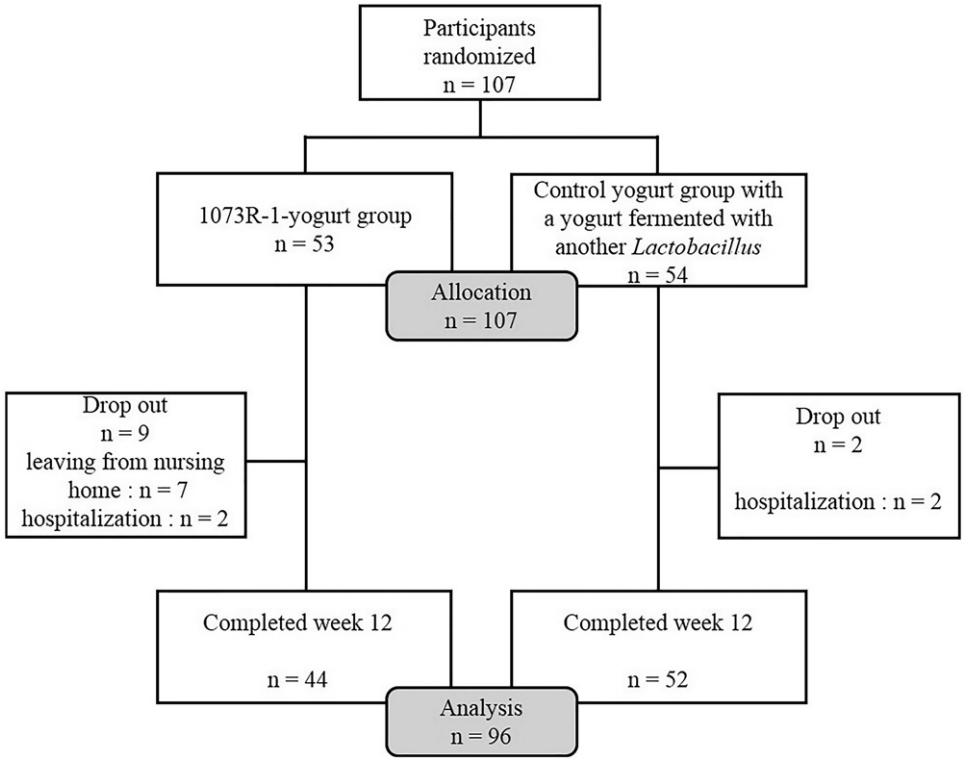

Figure 1. Flow chart showing an overview of participants and groupings in the randomized trial.

and Meiji Co., Ltd. in 2014 (approval number: 33), and conformed to the principles outlined in the Declaration of Helsinki of 1975, as revised in 2013. At the start of the trial, all participants and their families were given written and verbal interpretations regarding the aims and methods of the trial. In addition, potential risks, benefits and procedures to protect personal information with freedom of consent and withdrawal were clearly explained. All participants (or their families, where applicable) signed informed consent forms before the study. This study was registered with the Clinical Trial Registry of the University Hospital Medical Information Network Clinical Trials Registry (ID: UMIN000028636).

This clinical trial was targeted at elderly residents of nursing homes, who experienced reduced immunity, dysfunction of swallowing and high incidence of aspiration pneumonia [27]. Therefore, because of ethical considerations, we could not use test foods other than yogurt, such as milk, for the control group. Therefore, we used the same yogurt fermented with a different *Lactobacillus* strain, like the one available in the market at the time of the study.

Experimental design

The trial followed a simultaneous comparative design to compare the consumption of 1073R-1-yogurt and yogurt fermented with a different *Lactobacillus* strain, *L. bulgaricus* OLL1256. The safety of *L. bulgaricus* OLL1256-fermented yogurt to humans has been established, and the yogurt is commercially available. Previously, it has been established that the effect of consuming yogurt fermented with *L. bulgaricus* OLL1256 on NK cell activation is not different from that of consuming water and unfermented milk and, therefore, *L. bulgaricus* OLL1256-fermented yogurt was used as the control yogurt in this study [28].

The randomization, assignment and blinded tests of the participants as well as the production and management of test yogurts were all performed by independent specialists. An administrator of a plant manufacturing yogurt, who was independent of this study, listed one of the two alphabets P or Q on the test yogurt lids at the manufacturing plant to identify the two types of yogurt. Refrigerated yogurts were delivered to the two nursing homes once a week and stored in a refrigerator until consumption. The test yogurt P was distributed to one group of participants, while the test yogurt Q was distributed to the other group at every meal table by the care staff every day. The type and intake of yogurt ingested by the participants were confirmed daily by the nursing staff.

The methods of this work are based on the methods used in our previous research with yogurt [25]. Participants consumed 100 g of fermented yogurt every day between 10:00 and 10:30 am, for 12 weeks. During the period of study, participants avoided the intake of yogurt other than those included in the study. All participants resided in one of two nursing homes, with their meal constituents, mealtimes (breakfast at 8:00 am), wake-up time (6:00 to 7:00 am) and bed-time (9:00 pm) consistent between the two sites. The daily calorie ingestion of residents was about 1600 kcal. The room air temperature of the two nursing homes was maintained at 22 °C. Saliva sample was collected 1 h after breakfast (9:30–10:00 am), before (0 weeks) and after 4, 8 and 12 weeks of yogurt consumption.

Diets

Yogurt is defined as a clotted milk product that results from the fermentation of lactic acid in milk by *L. bulgaricus* and *Streptococcus thermophilus* [29]. Furthermore, at the time of manufacture, the finished product must include more than 10^8 live *L. bulgaricus* cells per gram, and the bacterial activity must be preserved during the entire shelf life, as confirmed through an appropriate activity test [30].

The yogurts used in this trial were produced by Meiji Co., Ltd. (Tokyo, Japan) with the 1073R-1-yogurt prepared using the lactic acid bacterial strains *L. bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 [23]. The control yogurt was prepared using the lactic acid bacterial strains *L. bulgaricus* OLL1256 and *S. thermophilus* OLS3295 [21]. Meiji Co., Ltd. acknowledges that *S. thermophilus* OLS3295 coexists with *L. bulgaricus* OLL1256 successfully and that fermentation is successful with this stain of *S. thermophilus*. These strains were initially isolated from traditional Bulgarian yogurt. The cell counts of *L. bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 in the 1073R-1-yogurt were between 1.8 and 3.5×10^8 colony-forming units (CFU) g^{-1} and 8.1–10.8×10^8 CFU g^{-1}, respectively. Likewise, the cell counts of *L. bulgaricus* OLL1256 and *S. thermophilus* OLS3295 in the control yogurt were approximately 1.7×10^8 CFU g^{-1} and 2.6×10^8 CFU g^{-1}, respectively. The two kinds of yogurt were also indistinguishable in taste. Both yogurts are readily available in the market, and their safety to humans has been established.

Saliva collection

Saliva of the elderly participants was collected using the protocol described in a previous study [25]. The saliva of participants was collected by a dentist or a dental hygienist using a Salikids saliva-collecting tool (Sarstedt AG & Co., Nümbrecht, Germany).

Measurement of total IgA levels

The total concentration of IgA in the saliva was assessed with a human IgA ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, USA), as previously described [25].

The saliva flow rate (mL min^{-1}) was defined by dividing the saliva weight (mg) by the gathering time, under the assumption that the saliva specific weight was 1.00 g mL^{-1}. The salivary IgA flow rate (μg min^{-1}) was computed by multiplying the absolute IgA concentration (μg mL^{-1}) by the saliva flow rate (mL min^{-1}) [25].

Measurement of influenza virus-bound IgA titres

Influenza virus-bound IgA titres were quantitated by ELISA using the human IgA ELISA quantification kit (Bethyl

Laboratories, Montgomery, TX). Biotinylated vaccines for influenza A virus subtypes, H3N2 and H1N1 and influenza B virus were prepared at Kitasato Institute for Life Sciences, Kitasato University (Tokyo, Japan), as previously described [31]. Ninety-six well microplates were coated with goat anti-human IgA diluted to 1:100 with coating-buffer (0.05 mol L^{-1} carbonate-bicarbonate, pH 9.6) for 1 h at 25 °C. These were then washed 5 times with wash-solution (50 m mol L^{-1} Tris, 0.14 mol L^{-1} NaCl, 50 g L^{-1} Tween 20, pH 8.0), and blocked with blocking solution (50 m mol L^{-1} Tris, 0.14 mol L^{-1} NaCl, 10 g L^{-1} bovine serum albumin [BSA], pH 8.0) for 30 min at 25 °C. After washing with wash-solution, test samples were placed in each well and allowed to stand for 1 h at 25 °C. After another round of washing, 1 μg mL^{-1} of biotinylated influenza A virus subtype H3N2, H1N1 or influenza B virus vaccine was added in blocking solution (50 m mol L^{-1} Tris, 0.14 mol L^{-1} NaCl, 10 g L^{-1} BSA, pH 8.0) to each well and left for 1 h at 25 °C. After a final washing step, 1:1000 dilutions of streptavidin-horseradish peroxidase conjugate (Merck Millipore, Darmstadt, Germany) were put in each well and left for 1 h at 25 °C. The reactions were developed for 15 min with 3,3′,5,5′-tetramethylbenzidine substrate in the dark and then stopped with 0.18 mol L^{-1} H_2SO_4. Absorbance was determined at 450 nm using an absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA). Influenza virus-bound IgA titres were determined with a standard curve.

Statistical analysis

Data are expressed as means with standard error (SE). Statistical differences in the baseline characteristics between the two groups, including body weight and body mass index (BMI) before, during and after 12 weeks of yogurt consumption, were analysed using Welch's *t*-tests. The effects of the duration of yogurt consumption on saliva flow rate, total salivary IgA and total IgA flow rate were analysed using regression analysis. Statistical differences were inferred by assessing the differences in the slopes of the regression lines. Influenza virus-bound IgA levels were analysed using factorial two-way analysis of variance (ANOVA) after logarithmic transformation. *p* Values less than .05 were considered to indicate statistically significant differences between results.

Results

Baseline condition

Table 1 shows the characteristics of participants between 1073R-1-yogurt and control yogurt groups at baseline (week 0). Owing to initial randomization, there were no differences between the two groups in any of these baseline characteristics.

Changes in body weight and body mass indices (BMI) after consumption of the study yogurts

There were no significant differences in body weight or BMI before, during or after 12 weeks of consumption in the 1073R-1- and control yogurt groups ($p = 1.0$ and .9, respectively; Table 2).

Changes in the saliva flow rate, and total IgA concentration and flow rate after consumption of the study yogurts

We observed an increase in the saliva flow rate ($p = .04$; Figure 2(a)), concentration of total IgA ($p = .04$; Figure 2(b)) and total IgA flow rate ($p = .002$; Figure 2(c)) in association with the duration of yogurt intake in both the 1073R-1 and control yogurt groups. There were no significant differences between the slopes of the regression lines between the 1073R-1-yogurt and control groups for saliva flow rate ($p = 1.0$; Figure 2(a)), concentration of total IgA ($p = .9$; Figure 2(b)) or total IgA flow rate ($p = .9$; Figure 2(c)).

Changes in influenza virus-bound IgA levels in saliva after consumption of the study yogurts

We found that there was no interaction between yogurt type and intake duration that affected total influenza virus-bound IgA levels (interaction: $p = 1.0$; Figure 3). However, when different virus subtypes were analysed individually, the different yogurt types affected influenza A virus subtype H3N2-bound IgA levels in participant saliva ($p = .001$; Figure 3(b)). Furthermore, the influenza A virus subtype H3N2-bound IgA levels in the 1073R-1-yogurt group were higher than those in the control yogurt group (Figure 3(b)). There were no similar effects for influenza A virus subtype H1N1-bound IgA or

Table 1. Baseline characteristics of subjects ($n = 107$).

	1073R-1-yogurt group ($n = 54$)		Control yogurt group, consuming yogurt fermented with a different *Lactobacillus* strain ($n = 53$)		*p* Value
	Mean	SE	Mean	SE	
Age (years)	86.3	1.15	88.4	0.81	.2
Sex (male/female)	18/36	–	15/38	–	–
Body weight (kg)	46.9	1.05	45.7	1.53	.5
BMI (kg m^{-2})	20.7	0.35	20.0	0.53	.3
Care required level (1–5)	3	–	3	–	–
Dementia level (I–M)	IIIa	–	IIIa	–	–

No significant differences were detected between groups across any of the baseline characteristics (Welch's *t*-test).

Table 2. Changes in body weight and BMI after yogurt consumption ($n = 96$).

	1073R-1-yogurt group ($n = 44$)				Control yogurt group, consuming yogurt fermented with a different *Lactobacillus* strain ($n = 52$)			
	Before yogurt intake		After 12 weeks of yogurt intake		Before yogurt intake		After 12 weeks of yogurt intake	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body weight (kg)	46.9	1.55	47.2	1.57	45.9	1.02	45.9	1.04
BMI (kg m^{-2})	20.8	0.54	20.9	0.55	20.2	0.36	20.1	0.35

No significant differences in the mean body weight and BMI were observed before or after 12 weeks of yogurt intake between the 1073R-1 and control yogurt groups ($p = .9$ and .9, 1.0 and .9, respectively, Welch's *t*-test).

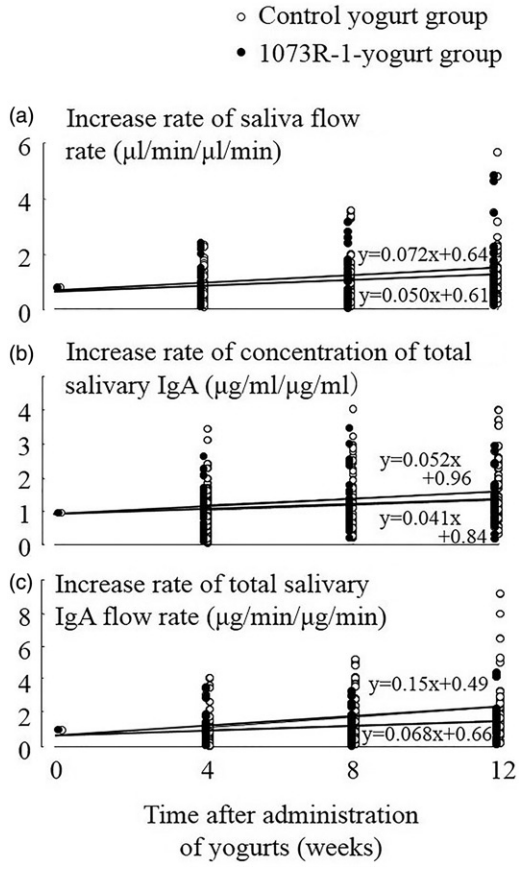

Figure 2. Effects of consuming 1073R-1-yogurt ($n = 44$) or control yogurt ($n = 52$), and duration of intake (a) on the saliva flow rate, (b) concentration of total salivary IgA and (c) total salivary IgA flow rate. The observed increase in the saliva flow rate, the concentration of total salivary IgA and total salivary IgA flow rate positively correlated with the duration of yogurt intake ($p = .002$ and .04, .02 and .04, <.001 and .002, respectively, regression analysis). There were no differences between the slopes of the regression lines between the 1073R-1 and control yogurt groups ($p = 1$, .9, .9, respectively, test for the difference of slope of the regression line).

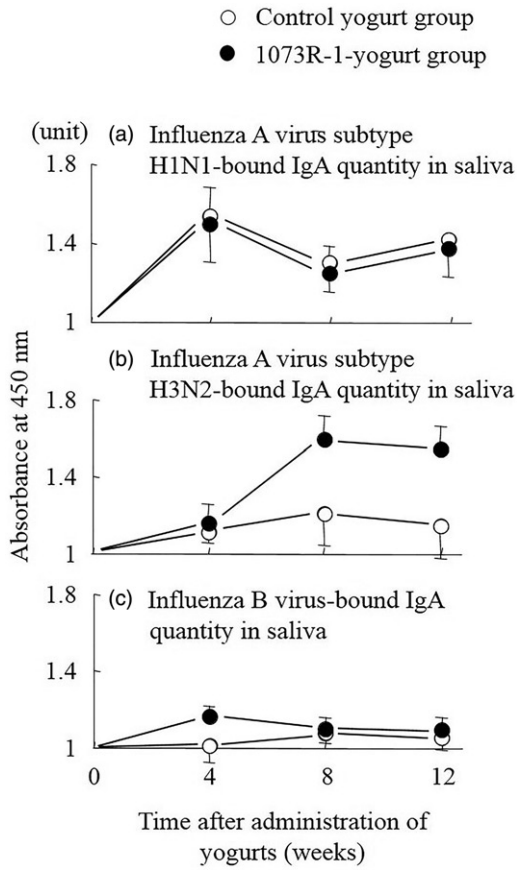

Figure 3. Effects of consuming 1073R-1-yogurt ($n = 44$) or control yogurt ($n = 52$), and duration of intake on the levels of (a) influenza A virus subtype H1N1-bound IgA quantity in saliva, (b) influenza A virus subtype H3N2-bound IgA quantity in saliva and (c) influenza B virus-bound IgA. Means and standard errors are shown. There was no interaction between yogurt type, duration of intake and IgA levels (interaction: $p = 1$, .4, .6, respectively, two-way ANOVA). However, the yogurt type affected influenza A virus subtype H3N2-bound IgA levels in participant saliva ($p = .001$; (b)). Furthermore, influenza A virus subtype H3N2-bound IgA levels in the 1073R-1-yogurt group were higher than those in the control group (b). Similar effects were not observed for influenza A virus subtype H1N1-bound IgA or influenza B virus-bound IgA ($p = .4$ and .2, respectively; (a,c)).

influenza B virus-bound IgA ($p = .4$ and .2, respectively; Figure 3(a,c)).

Discussion

Saliva and salivary IgA both play vital roles in protecting the oropharyngeal and upper airway region from diseases such as URTI and influenza virus infection [1,32,33], although both saliva flow and IgA levels have been shown to decline with age [33]. The decrease likely contributes to increased incidences of respiratory diseases and influenza virus infection in elderly people.

Effects of yogurt consumption on the saliva flow rate

Our study has shown that the consumption of 1073R-1-yogurt may contribute to increasing the saliva flow and IgA levels in elderly people, thereby bolstering their immune

systems. More specifically, we used a single regression analysis to show that the saliva flow rate increased relative to the intake duration of fermented yogurt; however, there were no significant differences between the 1073R-1 and control yogurt groups (Figure 2(a)). Previously, Finkel and Holbrook have reported that reactive oxygen species are involved in the aging process and that antioxidant activity decreases with age [34]. In addition, studies using a rat model revealed that high protein intake increases total oxidant status and oxidative stress indices in salivary glands. Consequently, the unstimulated salivary flow rate was significantly lower in a high oxidant group compared to controls [35]. Supporting a possible link between saliva flow and yogurt consumption, several studies have reported that *Lactobacillus ssp*. ingestion can increase the total antioxidant capacity of both mice and humans [36–38]. Based on the results of this study, we hypothesize that yogurt fermented with *Lactobacillus ssp*. may have acted to increase the total antioxidant capacities of the elderly volunteers, resulting in an increased flow rate of saliva that depends on the duration of intake. As it has been previously reported that saliva flow rate decreases with age [39], and the intake of yogurts fermented with *Lactobacillus* may contribute to increasing the saliva flow rate. This would also aid in reducing issues associated with dry mouth that are common in the elderly population, such as decreased mucosal immunity and increased dental caries [40].

Effects of yogurt intake on total salivary IgA level

Our experiments and regression analysis also showed that total IgA concentration and saliva flow rate increased relative to the duration of intake. Again, there were no differences between the 1073R-1-yogurt and control yogurt groups (Figure 2(b,c)). In our previous before-after non-randomized intervention study, total salivary IgA levels were found to increase after consumption of 1073R-1-yogurt [25]. This study extends this observation using a multi-centre, double-blind, parallel-group, randomized controlled trial to confirm that ingestion of 1073R-1-yogurt increased total IgA concentration and flow rate to levels similar to that observed upon ingestion of yogurt fermented with a different *Lactobacillus* strain. Previous studies have reported that the oral ingestion of *Lactobacillus* stimulated the gut mucosal immune system, and increased cytokine production and IgA^+ B cell expression in the small intestine [41]. In this study, it can be speculated that the *Lactobacillus* present in yogurt may also activate the gut mucosal immune system and increase the expression levels of cytokines in the gut. As a result, the salivary IgA secretion might be accelerated by the increase in the number of Ig^+ B cells mediated by cytokines in the small intestine. It is generally assumed that immune function declines with age, and compared with younger people, elderly people are less resistant to pathogenic microbes [42]. Therefore, the consumption of 1073R-1-yogurt may increase total IgA levels of saliva, bolstering immunity in elderly people and protecting against infection.

Effects of consumption of R-1-yogurt on the influenza virus-bound IgA levels in saliva

Finally, two-way ANOVAs showed that influenza A virus subtype H3N2-bound IgA levels in saliva were increased specifically upon consumption of 1073R-1-yogurt when compared with that by the control yogurt (Figure 3(b)). It has been reported that *L. bulgaricus* OLL1073R-1 is a *Lactobacillus* strain exhibiting high yields of EPS [43]. It has been revealed that the amount of EPS production in *L. bulgaricus* OLL1073R-1 is approximately twice that in *L. bulgaricus* OLL1256 [43]. Previously, Nagai et al. reported that 1073R-1-yogurt and EPS produced by *L. bulgaricus* both increased survival when orally ingested by BALB/c mice for 21 d before nasal infection with influenza A virus subtype H1N1. Moreover, influenza virus titers were decreased in the experimental groups relative to water controls, and there were also increased levels of anti-influenza virus IgA in bronchoalveolar lavage fluid (BALF) 4 d after infection [31]. Additionally, Matsuzaki et al. showed that co-addition of mouse Peyer's patch cells with EPS produced by *Leuconostoc mesenteroides* ssp. *mesenteroides* strain NTM048 and influenza A subtype H1N1 virus antigen elicited H1N1-antigen-specific IgA production [44]. However, this study is the first to report that influenza virus-bound IgA level of saliva increased after continuous intake of 1073R-1-yogurt. Tsai et al. conducted a mechanistic study and found that enhanced IgA production, *via* a T cell dependent pathway, induces the production of highly compatible IgA that, owing to somatic hypermutation, are specific for the target antigen. This course is responsible for affinity-based maturity according to the upregulation of TGF-β1, IL-21 and retinoic acid [45]. We, therefore, hypothesize that *L. bulgaricus* OLL1073R-1 EPS may induce production of high-affinity IgA specific to influenza virus through a pathway of T cell dependency that upregulates TGF-β1, IL-21 and retinoic acid.

In this study, although we found that the consumption of 1073R-1-yogurt increased influenza A virus subtype H3N2-bound IgA level in the saliva, influenza A virus subtype H1N1-bound IgA and influenza B virus- bound IgA did not show a similar increase (Figure 3(a,c)). Previously, Nagai et al. reported that oral administration of *L. bulgaricus* OLL1073R-1 EPS could enhance anti-influenza virus antibody titres in the BALF of mice nasally infected with influenza virus [31]. In addition, influenza A subtype H3N2 was the most prevalent strain found in Japan at the time of this trial, between October and December 2014 [46]. Therefore, it is more likely that the elderly volunteers would have been sensitized to the influenza A virus subtype. As such, *L. bulgaricus* OLL1073R-1 EPS may serve as an effective adjuvant for the sensitization of influenza virus antigen through the mucosa, thereby increasing only the levels of the influenza A virus subtype H3N2-bound IgA. Nevertheless, influenza infection-related complications are important causes of hospitalization and mortality in the elderly population [47]. Therefore, to effectively prevent mucosal infection, the induction of a strong influenza virus-bound IgA response is crucial. Based on our data, we believe that the continuous daily consumption of 1073R-1-yogurt would contribute to maintaining an

effective IgA response, combating influenza infection in the elderly population.

Consideration

To our knowledge, this study is the first to report that the salivary influenza virus-bound IgA level increases in older adults regularly consuming 1073R-1-yogurt. However, discretion is required when interpreting our results owing to a few limitations. First, our study did not incorporate a yogurt non-consumption group as a control. This clinical trial was targeted at elderly residents of nursing homes, who had reduced immunity, dysfunction of swallowing and high incidence of aspiration pneumonia; therefore, because of ethical considerations, we could not use test foods other than yogurt, such as milk, for the control group. Hence, we could not eliminate the potential effect of yogurts on the flow rate of saliva and salivary IgA in general. Second, we could not use the same *S. thermophilus* strains for 1073R-1-yogurt and control yogurt, because, for successful fermentation, the strains of *S. thermophilus* required temperatures different from that required by the two *Lactobacillus* strains. However, it must be emphasized that the purpose of our study was not to study the effect of *Lactobacillus* and *S. thermophilus* but to investigate the effect of consuming 1073R-1-yogurt. In addition, the immunostimulatory effect by *S. thermophilus* OLS 3059 has not yet been reported. Makino et al. compared the immunostimulatory activity of yogurt fermented with *L. bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 with that of yogurt fermented with *L. bulgaricus* OLL1245 and *S. thermophilus* OLS3059 and revealed that an enhanced immunoreactivity was observed only with yogurt fermented with *L. bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 [43]. Therefore, it might be not *S. thermophilus* OLS3059 but *L. bulgaricus* OLL1073R-1 that affected influenza A virus subtype H3N2-bound IgA levels.

Conclusion

In conclusion, our data indicate that consumption of 1073R-1-yogurt increases influenza A virus subtype H3N2-bound IgA levels in the saliva of elderly population living in Japanese nursing homes. Furthermore, we observed an increase in saliva flow rate, IgA concentration in saliva and IgA flow rate in saliva, which depended on the duration of yogurt intake in both 1073R-1 and control yogurt groups. Taken together, these results indicate that the consistent, daily intake of 1073R-1-yogurt may help prevent URTI and influenza A virus subtype H3N2 infection in elderly people with reduced immunity.

Acknowledgements

We thank the staff and residents of the nursing homes, 'Samukawa Home' and 'Honobono', for their support and participation.

Disclosure statement

This study was conducted in collaboration with Meiji Co., Ltd. The yogurts used in this study were produced by Meiji Co., Ltd. The funder had no role in data collection and interpretation, or the decision to submit the study for publication.

Funding

This study was funded in part by Meiji Co., Ltd. This study was supported by JSPS KAKENHI [Grant Numbers 15H06809, 17K11694].

References

[1] Enberg N, Alho H, Loimaranta V, et al. Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001;92:292–298.

[2] Grimoud AM, Arnaud C, Dellamonica P, et al. Salivary defence factor concentrations in relation to oral and general parameters in HIV positive patients. Eur J Oral Sci. 1998;106:979–985.

[3] Llena-Puy C. The rôle of saliva in maintaining oral health and as an aid to diagnosis. Med Oral Patol Oral Cir Bucal. 2006;11: E449–E455.

[4] Muddugangadhar BC, Sangur R, Rudraprasad IV, et al. A clinical study to compare between resting and stimulated whole salivary flow rate and pH before and after complete denture placement in different age groups. J Indian Prosthodont Soc. 2015;15: 356–366.

[5] Fagarasan S, Honjo T. Intestinal IgA synthesis: regulation of front-line body defences. Nat Rev Immunol. 2003;3:63–72.

[6] Bårnes GK, Workalemahu B, Kristiansen PA, et al. Salivary and serum antibody response against Neisseria meningitidis after vaccination with conjugate polysaccharide vaccines in Ethiopian volunteers. Scand J Immunol. 2016;84:118–129.

[7] Heineke MH, van Egmond M. Immunoglobulin A: magic bullet or Trojan horse? Eur J Clin Invest. 2017;47:184–192.

[8] Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol. 2009;70:505–515.

[9] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005;11:S45–S53.

[10] Nieman DC, Henson DA, Gusewitch G, et al. Physical activity and immune function in elderly women. Med Sci Sports Exerc. 1993; 25:823–831.

[11] Costabile A, Bergillos-Meca T, Rasinkangas P, et al. Effects of soluble corn fiber alone or in synbiotic combination with *Lactobacillus rhamnosus* GG and the pilus-deficient derivative GG-PB12 on fecal microbiota, metabolism, and markers of immune function: a randomized, double-blind, placebo-controlled, cross-over study in healthy elderly (Saimes study). Front Immunol. 2017;8:1443.

[12] Chung JY, Hsu CC, Chen JH, et al. Geriatric influenza death (GID) score: a new tool for predicting mortality in older people with influenza in the emergency department. Sci Rep. 2018;8:9312.

[13] Vangeti S, Yu M, Smed-Sörensen A. Respiratory mononuclear phagocytes in human influenza a virus infection: their role in immune protection and as targets of the virus. Front Immunol. 2018;9:1521.

[14] Sheu SM, Tsai CF, Yang HY, et al. Comparison of age-specific hospitalization during pandemic and seasonal influenza periods from 2009 to 2012 in Taiwan: a nationwide population-based study. BMC Infect Dis. 2016;16:88.

[15] FAO/WHO. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. 2001 Oct 1–4; Cordoba, Argentina.

[16] Seddik HA, Bendali F, Gancel F, et al. *Lactobacillus plantarum* and its probiotic and food Potentialities. Probiotics Antimicrob Proteins. 2017;9:111–122.
[17] Gupta V, Nag D, Garg P. Recurrent urinary tract infections in women: how promising is the use of probiotics? Indian J Med Microbiol. 2017;35:347–354.
[18] Ezendam J, van Loveren H. Probiotics: immunomodulation and evaluation of safety and efficacy. Nutr Rev. 2006;64:1–14.
[19] Harbige LS, Pinto E, Allgrove J, et al. Immune response of healthy adults to the ingested probiotic *Lactobacillus casei* Shirota. Scand J Immunol. 2016;84:353–364.
[20] Kotani Y, Shinkai S, Okamatsu H, et al. Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial. Immun Ageing. 2010;7:11.
[21] Arena MP, Caggianiello G, Russo P, et al. Functional starters for functional yogurt. Foods. 2015;4:15–33.
[22] Lee A, Lee YJ, Yoo HJ, et al. Consumption of dairy yogurt containing *Lactobacillus paracasei* ssp. *paracasei, Bifidobacterium animalis* ssp. *lactis* and heat-treated *Lactobacillus plantarum* improves immune function including natural killer cell activity. Nutrients. 2017;9:pii:558.
[23] Makino S, Ikegami S, Kume A, et al. Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. Br J Nutr. 2010;104:998–1006.
[24] Nagai T, Makino S, Ikegami S, et al. Effects of oral administration of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 and its exopolysaccharides against influenza virus infection in mice. Int Immunopharmacol. 2011;11:2246–2250.
[25] Yamamoto Y, Fujino K, Saruta J, et al. Effects of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 on the IgA flow rate of saliva in elderly persons residing in a nursing home: a before-after non-randomised intervention study. Gerodontology. 2017;34:479–485.
[26] Ministry of Health, Labour and Welfare. About the promotion of the dementia measures in Japan [monograph on the Internet]. [cited 2019 Mar 19]. Available from: https://www.mhlw.go.jp/file/05-Shingikai-12601000-Seisakutoukatsukan-Sanjikanshitsu_Shakaihoshoutantou/0000021004.pdf
[27] Kikutani T, Tamura F, Tashiro H, et al. Relationship between oral bacteria count and pneumonia onset in elderly nursing home residents. Geriatr Gerontol Int. 2015;15:417–421.
[28] Makino S, Ikegami S, Kano H, et al. Immunomodulatory effects of polysaccharides produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. J Dairy Sci. 2006;89:2873–2881.
[29] Bourlioux P, Pochart P. Nutritional and health properties of yogurt. World Rev Nutr Diet. 1988;56:217–258.
[30] Adolfsson O, Meydani SN, Russell RM. Yogurt and gut function. Am J Clin Nutr. 2004;80:245–256.
[31] Nagai T, Kataoka E, Aoki Y, et al. Alleviative effects of a Kampo (a Japanese Herbal) medicine "Maoto (Ma-Huang-Tang)" on the early phase of influenza virus infection and its possible mode of action. Evid Based Complement Alternat Med. 2014;2014:187036.
[32] McFarlin BK, Carpenter KC, Davidson T, et al. Baker's yeast beta glucan supplementation increases salivary IgA and decreases cold/flu symptomatic days after intense exercise. J Diet Suppl. 2013;10:171–183.
[33] Ichinohe T, Ainai A, Ami Y, et al. Intranasal administration of adjuvant-combined vaccine protects monkeys from challenge with the highly pathogenic influenza A H5N1 virus. J Med Virol. 2010;82:1754–1761.
[34] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408:239–247.
[35] Kołodziej U, Maciejczyk M, Niklińska W, et al. Chronic high-protein diet induces oxidative stress and alters the salivary gland function in rats. Arch Oral Biol. 2017;84:6–12.
[36] Mahdavi R, Nikniaz L, Ostadrahimi A, et al. Lactobacillus intake for 60 days favors antioxidant status of human breast milk: an RCT. Asia Pac J Clin Nutr. 2017;26:619–623.
[37] Li X, Wang N, Yin B, et al. Effects of *Lactobacillus plantarum* CCFM0236 on hyperglycaemia and insulin resistance in high-fat and streptozotocin-induced type 2 diabetic mice. J Appl Microbiol. 2016;121:1727–1736.
[38] Akkasheh G, Kashani-Poor Z, Tajabadi-Ebrahimi M, et al. Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double-blind, placebo-controlled trial. Nutrition. 2016;32:315–320.
[39] Tanida T, Ueta E, Tobiume A, et al. Influence of aging on candidal growth and adhesion regulatory agents in saliva. J Oral Pathol Med. 2001;30:328–335.
[40] López R, Smith PC, Göstemeyer G, et al. Ageing, dental caries and periodontal diseases. J Clin Periodontol. 2017;44 Suppl 18:S145–S152.
[41] Galdeano CM, Perdigón G. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol. 2006;13:219–226.
[42] Akimoto T, Kumai Y, Akama T, et al. Effects of 12 months of exercise training on salivary secretory IgA levels in elderly subjects. Br J Sports Med. 2003;37:76–79.
[43] Makino S, Sato A, Goto A, et al. Enhanced natural killer cell activation by exopolysaccharides derived from yogurt fermented with Lactobacillus delbrueckii ssp. bulgaricus OLL1073R-1. J Dairy Sci. 2016;99:915–923.
[44] Matsuzaki C, Hayakawa A, Matsumoto K, et al. Exopolysaccharides produced by *Leuconostoc mesenteroides* strain NTM048 as an immunostimulant to enhance the mucosal barrier and influence the systemic immune response. J Agric Food Chem. 2015;63:7009–7015.
[45] Tsai YT, Cheng PC, Pan TM. The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. Appl Microbiol Biotechnol. 2012;96:853–862.
[46] Ministry of Health, Labour and Welfare. About the occurrence situation of Influenza in Japan [monograph on the Internet]. [cited 2019 Mar 19]. Available from: http://www.mhlw.go.jp/file/06-Seisakujouhou-10900000-Kenkoukyoku/0000200441.pdf
[47] Freitas ARR, Donalisio MR. Excess of mortality in adults and elderly and circulation of subtypes of influenza virus in Southern Brazil. Front Immunol. 2018;8:1903.