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Isolation and characterization of probiotic strains for improving oral health

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ABSTRACT

Probiotics have been proven effective for preventing caries. In contrast, the effect of probiotics on improving oral diseases such as gingivitis, periodontitis and halitosis has been less explored.

Objective: To perform a screening of lactic acid bacteria, according to international guidelines for the evaluation of probiotics, in order to select candidate probiotic strains for preventing oral disorders.

Study design: The strains were isolated from healthy children and were subjected to a variety of *in vitro* tests in order to show their functionality. The safety of the strains was assessed by determining antibiotic susceptibility and production of lactic acid.

Results: Forty-six of the 100 new isolates were assigned to lactic acid bacteria genera after a biochemical characterization. Most of the new isolated strains seem to be resistant to oral conditions, have great ability to form aggregates and have high antagonistic activity against oral pathogens. None of the strains produced unpleasant volatile compounds. The strains showed high ability to adhere to oral tissues and they do not present any antibiotic resistance. Moreover, an increased risk of developing caries due to their ability to produce lactic acid was discarded in seven pre-selected probiotic candidates.

Conclusions: These lactic acid bacteria show promising properties to be used as potential probiotics for improving oral health.

1. Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit for the host.[1] A number of potential benefits arising from the use of probiotics have been demonstrated, including increased resistance to infectious diseases,[2,3] alleviation of lactose intolerance,[4] prevention of gut diseases, diarrhoea and vaginal and urogenital infections,[5,6] reduced allergy and respiratory infections,[7,8] reduced serum cholesterol concentration,[9] increased resistance to cancer chemotherapy and decreased risk of colon cancer.[10]

It has been stated that oral administration of probiotics may also benefit oral health by preventing the growth of harmful microbiota or by modulating mucosal immunity in the oral cavity.[11] However, probiotics have been historically poorly investigated from the perspective of oral health when compared with a gastrointestinal health point of view. This tendency has changed in the last decade and a number of studies have been performed in order to assess the effect of probiotics on oral health (Table 1). The impact of the oral administration

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Table 1 – Summary of the clinical trials aimed to evaluate the effect of probiotics on oral health.

Reference	Title	*n*	Dose/day	Vehicle	Endpoint	Results
Caries						
Näse et al.[12]	Effect of long-term consumption of a probiotic bacterium, *L. rhamnosus* GG, in milk on dental caries and caries risk in children	594	No data	Cheese	*S. mutans* level	Reduction of the number of microorganisms, especially in 3–4 years old children
Ahola et al.[13]	Short-term consumption of probiotic containing cheese and its effect on dental caries risk factors	74	240E+09	Cheese	*S. mutans* level, *Candida albicans* level, decayed missing filled teeth (DMFT), community periodontal index (CPI), number of healthy sextans	Reduction of *S. mutans* levels in all the subjects, regardless of the intervention group. Probiotic intervention might reduce the risk of the highest level of *S. mutans* and *C. albicans*.
Montalto et al.[21]	Probiotic treatment increases salivary counts of lactobacilli: a double-blind, randomized, controlled study	35	188E+09	Liquid and capsules	*S. mutans* level, lactobacilli level	Increment of lactobacilli, but no changes in *S. mutans* level regardless the group
Nikawa et al.[14]	*Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci	40	No data	Yoghourt	*S. mutans* level	Reduction of the number of *S. mutans* in the intervention group
Caglar et al.[18]	Effect of yoghourt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults	21	140E+10	Yoghourt	*S. mutans* level, lactobacilli level	Reduction of the number of *S. mutans* in the intervention group
Caglar et al.[15]	Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *L. reuteri* ATCC 55730 by straws or tablets	120	100E+08	Straw and tablets	*S. mutans* level, lactobacilli level	Reduction of the number of *S. mutans* in the intervention group
Caglar et al.[16]	Effect of chewing gums containing xylitol or probiotic bacteria on salivary mutans streptococci and lactobacilli	80	300E+08	Chewing gum	*S. mutans* level, lactobacilli level	Reduction of the number of *S. mutans* in the intervention group
Caglar et al.[17]	A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli	20	No data	Medical device	*S. mutans* level, lactobacilli level	Reduction of the number of *S. mutans* in the intervention group
Caglar et al.[19]	Short-term effect of ice-cream containing *B. lactis* Bb-12 on the number of salivary mutans streptococci and lactobacilli	24	530E+08	Ice-cream	*S. mutans* level, lactobacilli level	Reduction of the number of *S. mutans* in the intervention group
Stecksén-Blicks et al.[44]	Effect of long-term consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries and general health in preschool children: a cluster-randomized study	248	150E+09	Milk	caries increment	Daily consumption of milk containing probiotic bacteria and fluoride reduced caries in preschool children with a prevented fraction of 75%
Lexner et al.[45]	Microbiological profiles in saliva and supragingival plaque from caries-active adolescents before and after a short-term daily intake of milk supplemented with probiotic bacteria – a pilot study	18	250E+09	Milk	*S. mutans* level, lactobacilli level	No differences in the microbial profiles or levels of caries-associated bacteria in saliva and supragingival plaque samples collected from caries-active adolescents.

Chuang et al.[46]	Probiotic *Lactobacillus paracasei* effect on cariogenic bacterial flora	78	9E+08	Tablet	*S. mutans* level, lactobacilli level and salivary buffer capacity	A significant count reduction in the salivary *S. mutans* was detected between the completion of medication and two weeks after medication. Thus, a 2-week period of medication may be needed for *L. paracasei* to be effective in the probiotic action.
Candidiasis						
Hatakka et al.[24]	Probiotics reduce the prevalence of oral *Candida* in the elderly – a randomized controlled trial	276	150E+09	Cheese	*C. albicans* level	Probiotic intervention reduce the risk of high *Candida* counts
Halitosis						
Burton et al.[25]	A preliminary study of the effect of probiotic *S. salivarius* K12 on oral malodour parameters	23	>100E+09	Lozenge	Volatile sulphur compound (VSC) levels and microbiota composition	Bacteriocin-producing *S. salivarius* after oral antimicrobial mouthwash reduces oral VSC levels
Kang et al.[26]	Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds	46	100E+09	Oral rinse	Volatile sulphur compound (VSC)	*W. cibaria* isolates possess the ability to inhibit VSC production under both *in vitro* and *in vivo* conditions
Iwamoto et al.[47]	Effects of probiotic *Lactobacillus salivarius WB21* on halitosis and oral health: an open-label pilot trial.	20	200E+09	Tablet form	Organoleptic test, volatile sulphur compound (VSC) and bleeding on probing	Oral administration of probiotic lactobacilli primarily improved physiologic halitosis and also showed beneficial effects on bleeding on probing from the periodontal pocket
Gingivitis						
Krasse et al.[27]	Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*	59	200E+08	Chewing gum	Gingival index, plaque index, lactobacilli level	Reduction of gingivitis and plaque in patients with moderate to severe gingivitis
Twetman et al.[28]	Short term consumption of chewing gums containing probiotic *Lactobacillus reuteri* on the levels of inflammatory mediators in gingival crevicular fluid	42	200E+08	Chewing gum	Bleeding on probing, selected inflammatory mediators in gingival crevicular fluid	Improvement of bleeding on probing and reduction of pro-inflammatory cytokines
Staab et al.[48]	The influence of a probiotic milk drink on the development of gingivitis: a pilot study	50	No data	Milk	Papilla bleeding index, interproximal plaque and Turesky plaque index. Polymorphonuclear elastase, myeloperoxidase and matrix metalloproteinase-3 activities in gingival crevicular fluid	Improvement of elastase and metalloporteinase-3 activities
Harini and Anegundi[49]	Efficacy of a probiotic and chlorhexidine mouth rinses: a short-term clinical study	45	No data	Oral rinse	Gingival index, plaque index	Probiotic mouth rinse was found effective in reducing plaque accumulation and gingival inflammation.
Periodontitis						
Shimauchi et al.[30]	Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB 21: a randomized, double blind placebo controlled study	66	200E+09	Tablet	Probing pocket depth, gingival index, bleeding on probing, plaque index, salivary lactoferrin level, lactobacilli level	Improvement of periodontal clinical parameters and reduction of lactoferrin level, especially in smokers

Table 1 (*Continued*)

Reference	Title	*n*	Dose/day	Vehicle	Endpoint	Results
Zahradnik et al.[20]	Preliminary assessment of safety and effectiveness in humans of ProBiora^{3TM}, a probiotic mouthwash	20	1E+06 and 1E+08	Mouthwash	*Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythensis*, *Prevotella intermedia/ nigrescens*, *Porphyromonas gingivalis*, *Campylobacter rectus* levels	Reduce dental pathogens in saliva and periodontal pathogens in subgingival plaque
Tsubura et al.[50]	The effect of *Bacillus subtilis* mouth rinsing in patients with periodontitis	54		Mouth rinsing	Probing pocket depth, bleeding on probing, gingival index, and BANA test	Reduction of pathogen levels, improvement of gingival index, but not effect on probing pocket depth nor bleeding on probing
Mayanagi et al.[29]	Probiotic effects of orally administered *Lactobacillus salivarius* WB21-containing tablets on periodontophatic bacteria: a double-blinded, placebo-controlled, randomized clinical trial	66	200E+09	Tablet	*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* levels	There was no significant difference between probiotic and placebo groups in the direct count of any specific periodontopathic bacteria. However, the numerical sum of five selected periodontopathic bacteria in the test group was decreased significantly in subgingival plaque

of probiotics on dental caries has been studied in several experiments utilizing different strains. *Lactobacillus rhamnosus* GG alone or combined with other species,[12,17] *Bifidobacterium lactis* and a combination of three *Streptococcus* strains have proved their potential to reduce the number of *Streptococcus mutans* in saliva after a short period of consuming the probiotic.[18–20] Only one study failed in demonstrating an effect of a preparation containing several species of lactobacilli (Floragen®, Pharmamont, Italy) on reduction of *S. mutans* levels.[21]

Although a number of studies have demonstrated the ability of lactobacilli strains to inhibit *in vitro* oral pathogens different from *S. mutans*, little is known about the potential of probiotics for inhibiting these oral cariogenic pathogens in clinical trials.[22,23] Recently, Hatakka et al.[24] reported that *L. rhamnosus* GG reduces oral *Candida* counts in the elderly. Despite the potential of probiotics to prevent halitosis and breath odour by reestablishing an appropriate microbiota, this field is hardly unexplored. Burton et al.[25] demonstrated that bacteriocin-producing *Streptococcus salivarius* K12, after an oral antimicrobial mouthwash, reduces oral volatile sulphur compounds (VSC) levels and breath odour. Similarly, *Weissella cibaria* isolates in addition to inhibit *Fusobacterium nucleatum in vitro*, posses the ability to inhibit volatile sulphur compounds production under *in vivo* conditions.[26]

The putative beneficial effects of probiotics on gingivitis and periodontitis have also been evaluated. However, the studies show differing results depending on the strains used and the endpoints analysed (Table 1). For example, *Lactobacillus reuteri* can be used to reduce gingivitis and dental plaque in patients with moderate to severe gingivitis and also to reduce pro-inflammatory cytokines in gingival crevicular fluid.[27,28] However, *Lactobacillus salivarius* WB21 in tablets does not reduce the direct count of any specific periodontopathic bacteria – *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*,[29] even though this probiotic improves periodontal clinical parameters (probing pocket depth, gingival index, bleeding on probing and plaque index) especially in smoker subjects.[30] Therefore, it seems that further studies are needed in order to unambiguously demonstrate the effect of probiotics on periodontal diseases. These contradictory results point out that not all the probiotics, despite they have been used for other purposes, have beneficial effects on periodontal diseases. It seems necessary to perform specific screenings for selecting appropriate probiotic strains for preventing gingivitis or periodontitis. On the other hand, further studies are needed in order to explore the potential of probiotics for preventing halitosis. The aim of the present work was to isolate and characterize lactic acid bacteria, according to the international guidelines for the evaluation of probiotics, and to select strains with specific functionalities to be used as probiotics for reducing oral diseases, such as gingivitis, periodontitis or halitosis, for which probiotic therapy benefits are still unclear.

2. Materials and methods

2.1. Bacterial strains

Lactic acid bacteria (LAB) were isolated from saliva and faeces of healthy children up to 10 years old. The strains were grown

in different selective or differential culture media in order to isolate as much variability as possible within the lactic acid bacteria group. In spite of the fact that the culture media used favour the isolation of LAB strains, other species can also grow. In order to discard non-LAB strains, the isolated strains were biochemically characterized using the API 20 Strep and API 50 CHB commercial kits (Biomerieux España, Spain) following manufacturer's instructions.

S. salivarius K12 and *L. reuteri* strains were isolated from commercial tablets (BLIS Technologies Ltd., New Zealand) and GumPeriobalance® (BioGaia, Sweden), respectively. Strains of potentially pathogenic bacteria (*P. gingivalis* CIP 103683T, *F. nucleatum* CIP 104988, *T. denticola* CIP 103919, and *Prevotella denticola* CIP 104478T) were obtained from the Collection of Institute Pasteur. A hospital-isolated *S. mutans* wild-type strain was also used.

Lactic acid bacteria strains were grown in Man, Rogosa and Sharpe (MRS) plates for 24 h in microaerophilic conditions (5% CO_2). *P. gingivalis*, *F. nucleatum* and *P. denticola* pathogenic strains were grown in M20 medium (30 g tryptone, 20 g yeast extract, 0.5 g cysteine hydrochloride, 5 g glucose and 25 mL hemin solution (0.1 g hemin chloride, 4 mL triethanolamine and 96 mL distilled water) per litre) at 37 °C in anaerobic conditions. *T. denticola* was grown in M71 medium (15 g tryptone, 5 g yeast extract, 8 g di sodium hydrogen phosphate, 1 g ammonium carbonate, 0.5 g cysteine hydrochloride, 0.03 mL thioglycolic acid, 0.05 mL Tween 80 and 250 mL horse serum per litre) at 37 °C in anaerobic conditions, whilst *S. mutans* was grown in Tryptic Soy Agar (TSA) medium at 37 °C in aerobic conditions.

2.2. Survival at oral conditions

The ability to survive at oral conditions was assessed by inoculating 200 μL of MRS medium (Sigma–Aldrich Chem., Spain) supplemented with lysozyme (Sigma–Aldrich Chem., Spain) at 2×10^5, 4×10^5, 6×10^5 and 10^6 U/mL and hydrogen peroxide (Sigma–Aldrich Chem., Spain) at 1, 2.5, 5 and 15 mM with 5×10^7 cfu of each bacterial strain in 96-well culture plates. The initial lysozyme concentration was described in Iacono et al.,[31] this amount was multiplied by 2, 3 and 25 in order to study the survival of the strains in harder conditions. The initial hydrogen peroxide concentration was calculated taking in consideration that the strains could be in contact with a toothpaste containing 3% of hydrogen peroxide.[32] This amount was multiplied by 2.5, 5 and 15 times in order to test the survival of the strains in harder conditions. Plates were incubated at 37 °C and 5% CO_2 for 6 h. Bacterial growth was quantified by measuring optical density at 620 nm. Bacterial growth value was obtained by comparison with the growth achieved by the same strain in standard MRS medium without supplements. A global survival value (SV) was obtained as follows:

$$SV = \frac{LV + HV}{2}$$

where LV and HV are the averages of the bacterial growth values in the four different concentrations of lysozyme and hydrogen peroxide, respectively. This value is very informative since in addition to show if the strains are able to grow at oral conditions, it allows for classification of strains according to their resistance to these adverse conditions.

2.3. Formation of aggregates

The formation of aggregates or co-aggregates with other microorganisms is essential for bacteria to establish dental plaque or biofilm.[33,34] LAB probiotic strains with aggregation activity could inhibit or reduce dental plaque formation by pathogenic bacteria.[35] LAB strains were incubated overnight in MRS medium at 37 °C and 5% CO_2. Cells were resuspended in Phosphate Buffered Saline (PBS) after a wash with the same solution. The ability of probiotic candidates to form aggregates was evaluated by measuring the decrease of the optical density at 600 nm of the cell suspensions due to the formation of aggregates and precipitation. Aggregation capacity (AC) value was obtained using the following formula:

$$AC = \frac{1 - (OD_t/OD_0)}{100}$$

where OD_t and OD_0 are the optical density at final (6 h) and initial times, respectively.

2.4. Ability to antagonize pathogens

P. gingivalis CIP 103917T, *F. nucleatum* CIP 104988, *T. denticola* CIP 103919, *P. denticola* CIP 104478T; and wild-type *S. mutans* were used to evaluate antagonistic properties of the probiotic candidates. These pathogenic species were chosen because they are widely associated with gingivitis, periodontitis and halitosis in the literature. Probiotic candidates were grown on MRS plates at 37 °C and 5% CO_2 to confluence. Six millimetre diameter cylinder sections of the confluents were placed on M20 plates inoculated with *P. gingivalis, F. nucleatum* and *P. denticola* or M71 plates inoculated with *T. denticola* or TSA plates incubated with *S. mutans*. Plates were incubated overnight at 37 °C in anaerobic conditions, except for *S. mutans* which were incubated in aerobic conditions. Growth inhibitory activity (GI) was calculated subtracting the cylinder diameter (CD, mm) from the inhibition zone diameter (IZD, mm) as follows:

$$GI = \frac{IZD - CD}{2}$$

2.5. Production of malodour volatile compounds

The production of malodour volatile compounds by the probiotic candidate strains was determined when growing in a culture medium resembling human diet by means of a sensorial evaluation. Strains were grown in the medium which contained glucose (0.5%, w/v), fructose (0.5%, w/v), yeast extract (1%, w/v), meat extract (1%, w/v), eukaryotic cells (200 cells/mL) and pectin (0.5%, w/v) for 48 h at 37 °C and 5% CO_2. Three independent evaluators gave to each strain a production of malodour value (PMV) between 1 and 5 where 1 is absence of odour and 5 is a very unpleasant odour. Values shown are the average of three sensorial evaluations.

2.6. Ability to adhere to oral tissues

In vitro adhesion assay to pig tongue and Caco-2 cells, which could resemble oral gum, was carried out. Caco-2 cells were incubated in Roswell Park Memorial Institute medium

(RPMI-1640) in ELISA plates for 22 days, changing the culture media every 2 days. Each probiotic candidate strain was incubated overnight with 5-3H-tymidine (1.0 μCi/mL, Amersham Biosciences, UK)-labelled (10 μL/mL). The adhesion measurement was performed by the addition of 10^8 cfu-radio-labelled strains to 1.05 cm × 0.5 cm-fragments of pig tongue or pre-incubated Caco-2 cells in ELISA plates. After 45 min, supernatants were carefully removed, three washes with PBS were performed and the tongue or Caco-2 cells together with the adhering bacteria were scrapped. Recovered bacteria were lysed using 1% sodium dodecyl sulphate (SDS) in 0.1 mol/L NaOH by incubation at 60 °C for 1 h to calculate specific radioactivity. All assays were performed in triplicate.

2.7. Genetic bacterial identification

Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega Biotech Iberica, Spain). 16S gene was amplified by polymerase chain reaction (PCR) using bacteria universal primers. DNA was washed using QIAquick PCR Purification kit (Qiagene, Germany) and sequencing reactions were performed per sample, using primers fD1 and rP1described in Weisburg et al. and BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, USA), on a Genetic Analyzer 3130 (Applied Biosystems, USA).[36] DNA Sequence Analysis v.5.2 software (Applied Biosystems, USA) was used to collect data, which were analysed through Chromas (Technelysium Pty. Ltd.) and BioEdit (Ibis Biosciences, USA) software. Sequence databases (NCBI Reference Sequences – www.ncbi.nlm.nih.gov/RefSeq/, and Ribosomal Database Project – www.rdp.cme.msu.edu) were used for bacteria species identification.

2.8. Acid production

The ability to produce acid when growing in culture media supplemented with different sugars present in human diet was evaluated. The probiotic candidate strains were grown for 18 h at 37 °C and 5% CO_2 in the following media: MRS, minimal medium supplemented with 4% glucose, 4% fructose, 4% lactose, 4% sucrose or yeast extract at 15 g/L. The composition of the minimal medium per litre is: 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2{\cdot}6H_2O$, 2 g $NaHCO_3$, 0.05 g hemin, 0.5 g cysteine HCl, 0.5 g biliar salts, 2 g Tween 80 and 10 μL vitamin K1. Cultures were adjusted to pH 7 with HCl. The pH and the number of viable cells (cfu/mL) were measured at the end of the incubation time. Production of acid value (PAV) for each culture medium was obtained by multiplying pH by $\log_{cfu/mL}$. Based on these values; strains were classified from lower to higher lactic acid producing strains in each medium. In order to facilitate the interpretation of the results, a global PAV was obtained by calculating the average of the position of each strain in the different classifications.

2.9. Antibiotic susceptibility

The minimum inhibitory concentration (MIC) was determined for the following substances: kanamycin, streptomycin, quinuspristin + dalfopristin, ampicillin, tetracycline, chloramphenicol, clindamycin, erythromycin and gentamicin. Those antimicrobials were chosen to maximize the identification of resistance genotypes to the most commonly used antimicrobials by assessing the resistance phenotypes.[37]

Probiotics strains were grown at 37 °C and 5% CO_2 during 24 h in Landing Ship Medium (LSM) supplemented with the antimicrobials at different concentrations, except for gentamicin when the cells were incubated in Tryptic Soy Broth (TSB) medium to avoid interferences of the medium in the measurements. The growth was calculated by measuring optical density (OD) at 620 nm and comparing with the growth in the same medium without antimicrobials. The strains were categorized as susceptible or resistant to antimicrobials according to the breakpoints levels described recently by EFSA (European Food Safety Authority).[37]

2.10. Statistical methods

Survival at oral conditions, the ability to form aggregates for faecal and oral-isolated strains, and the adhesion ability to Caco-2 cells and pig tongue were compared using either Student's *t*-test or Mann–Whitney rank sum test. The choice of test was made using SIGMASTAT (Jandel Scientific, San Rafael, CA) program according to the distribution of data. The differences were considered significant when $P < 0.05$.

3. Results

3.1. Bacterial isolation and biochemical identification

A total of 100 strains were selected due to their excellent growth in different selective media used for strain isolation. Seventy-seven strains were isolated from oral cavity whilst the remaining 23 were obtained from faeces. These strains were biochemically characterized using API systems in order to discard non lactic acid bacteria. Thirty-nine strains were assigned to lactic acid bacteria genera, *Lactobacillus* being the most represented genus (with 27 strains), followed by *Lactococcus*, *Pediococcus* and *Leuconostoc* (with 7, 3 and 2 strains, respectively). Moreover, for seven strains the biochemical methods used were not conclusive enough to discriminate amongst different lactic acid bacteria genera. These strains were also selected for the following steps. Twenty-eight of these strains were oral cavity and 18 faeces-isolated. The remaining 54 strains were discarded.

3.2. Specific biofunctionalities for improving oral health

The ability to survive at oral conditions was extremely variable amongst strains. The survival values ranged from 95.86 to −49.12% and from 120.89 to 3.37% of growth in the same medium without supplements for lysozyme and hydrogen peroxide, respectively (Supplementary Table 1). There were no statistically significant differences in resistance to lysozyme (51.43 and 35.74 median values, $P = 0.81$) nor hydrogen peroxide (26.07 and 22.43 median values, $P = 0.54$) between faecal and oral isolated strains.

The aggregation values ranged between 56 and 0, with a median value of 12.02 (Table 2). As above, no differences were

Table 2 – Ability to form aggregates and to produce malodour compounds of probiotic candidates. Pre-selected strains are shown in bold. AB1 to AB18 were isolated from faeces whilst AB19 to AB46 were oral cavity-isolated strains.

Strain	Aggregation value[a]	Production of malodour[b]	Strain	Aggregation value[a]	Production of malodour[b]
S. salivarius	16.67	1	AB23	12.50	1
L. reuteri	0.00	3	AB24	22.73	4
AB1	19.23	3	AB25	13.64	4
AB2	0.00	3	AB26	14.08	1
AB3	3.85	2	AB27	13.04	4
AB4	11.54	2	AB28	13.90	1
AB5	0.00	1	AB29	0.00	1
AB6	19.23	2	AB30	15.80	1
AB7	3.85	2	AB31	12.80	4
AB8	12.50	3	AB32	20.83	1
AB9	25.00	3	AB33	36.36	4
AB10	30.77	2	AB34	13.04	4
AB11	15.90	2	AB35	0.00	2
AB12	14.80	2	AB36	20.83	2
AB13	3.85	3	AB37	0.00	3
AB14	3.85	**2**	**AB38**	0.00	2
AB15	7.69	2	AB39	13.04	2
AB16	0.00	2	AB40	0.00	2
AB17	0.00	2	AB41	0.00	2
AB18	11.54	**1**	**AB42**	4.17	1
AB19	7.69	1	AB43	8.33	3
AB20	46.15	1	AB44	8.33	3
AB21	15.38	2	AB45	7.69	3
AB22	56.00	2	AB46	8.20	1

[a] Aggregation capacity (AC) is calculated as follows:$AC = \frac{1-(OD_t/OD_0)}{100}$
where OD_t and OD_0 are the optical density at 6 h and time 0, respectively.
[b] The strains received a value between 1 and 5 where 1 is absence of odour and 5 is a very unpleasant odour.

found in aggregation activity between oral and faecal strains (median value 12.92 and 9.62, $P = 0.423$).

The antagonistic activity results are shown in Table 3. It is notable that *P. gingivalis* was inhibited by only 7 out of the 46 strains. In contrast, *P. denticola*, *S. mutans*, *F. nucleatum*, and *T. denticola* were inhibited by 32, 29, 24, and 24 strains, respectively. Moreover, 11 of the strains that showed some kind of antagonism (45 strains) presented this activity against a single pathogen, whilst 8, 15 and 11 strains showed antagonism activity against 2, 3 and 4 pathogens, respectively (Table 3).

None of the strains evaluated received the highest value for the production of malodour volatile compound, discarding the

Table 3 – Antagonistic activity pattern of probiotic candidates against oral pathogens (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Treponema denticola*, *Prevotella denticola* and Streptococcus mutans). Pre-selected strains are shown in bold. AB1 to AB18 were isolated from faeces whilst AB19 to AB46 were oral cavity-isolated strains.

Strain	*Porphyromonas gingivalis*	*Fusobacterium nucleatum*	*Treponema denticola*	*Prevotella denticola*	*Streptococcus mutans*	Number of pathogens
S. salivarius, AB10, AB35, AB41, AB43	NI	NI	I	I	I	3
L. reuteri, AB25, AB36, AB37, AB43	NI	I	NI	I	I	3
AB1, AB45	I	I	NI	I	I	4
AB2, AB14, **AB15**	NI	NI	I	NI	I	2
AB3, **AB4**	NI	I	I	NI	I	3
AB5, AB13, AB16, AB17, **AB38**, **AB42**	NI	I	I	I	I	4
AB6, AB7, AB8, AB9, AB21, AB24, AB27, AB33	NI	NI	NI	I	NI	1
AB11	I	NI	NI	I	NI	2
AB12, AB28, AB31	NI	I	NI	NI	NI	1
AB18, AB34, AB39	NI	I	I	NI	I	3
AB19	I	I	NI	I	NI	3
AB20, AB32	NI	NI	NI	I	I	2
AB22	I	I	I	NI	I	4
AB23, AB26	NI	NI	NI	NI	NI	0
AB29	NI	NI	I	NI	NI	1
AB30	I	NI	I	I	I	4
AB40	NI	NI	I	I	NI	2
AB46	I	NI	I	I	NI	3

I, inhibition; NI, not inhibition.

Table 4 – Genetic identification, adhesion to pig tongue and Caco-2 cells and acid production of pre-selected probiotic strains. Strains grown in culture media containing sugars present in human diet (MRS, minimal medium supplemented with glucose (Glu), fructose (Fru), sucrose (Suc), lactose (Lac) and yeast extract (YE)).

Strain	Species	Isolated from	Adhesion (cfu/cm^2)		Acid production value						PA value[b]
			Caco-2	Tongue	MRS[a]	Glu[a]	Fruc[a]	Sac[a]	Lac[a]	YE[a]	
S. salivarius		Commercial product	8.83E+04	1.72E+06	41.37	49.19	55.67	57.39	54.01	71.04	1.83
L. reuteri		Commercial product	ND	ND	36.75	29.69	31.31	29.85	28.03	38.03	8
AB1	*Pediococcus acidilactici*	Faeces	2.50E+05	7.95E+06	34.85	30.87	33.09	33.40	33.03	50.00	7.17
AB4	*Pediococcus acidilactici*	Faeces	3.25E+04	1.96E+07	35.40	32.57	33.33	33.91	34.35	57.30	5.33
AB5	*Lactobacillus plantarum*	Faeces	5.19E+05	4.37E+07	34.57	31.68	33.51	34.86	33.21	56.59	6
AB15	*Lactobacillus plantarum*	Faeces	1.00E+06	8.84E+06	32.70	30.34	28.56	31.67	31.45	56.11	8.17
AB22	*Lactobacillus casei*	Saliva	8.06E+04	6.46E+06	36.39	32.31	33.63	59.69	36.64	61.76	3.83
AB38	*Lactobacillus brevis*	Saliva	3.78E+04	2.70E+06	42.04	48.28	45.24	59.12	58.24	62.53	1.83
AB42	*Pediococcus pentosaceus*	Saliva	1.71E+04	3.63E+06	41.59	39.67	37.34	55.52	38.73	62.60	2.83

[a] Production of acid is calculated by multiplying pH by $\log_{cfu/mL}$.
[b] Average of the position in the classifications of strains according to acid production in each culture media.

production of very unpleasant compounds. Twelve strains did not produce malodour; from them ten were oral isolated, whilst two were isolated from faeces (Table 2).

Before assessing the ability to adhere to oral tissues, a first pre-selection of the best candidate strains was made according to their functionalities; especially their antagonistic activity and their ability to form aggregates (see Section 4 for further details). The list of selected strains is shown in Table 4.

Adhesion capacity to Caco-2 cells and pig tongue of pre-selected strains was assessed by scintillation using tritium-labelled thymidine and compared to those of *S. salivarius* K12 commercial strain. In general, strains showed higher value of adhesion to tongue (values between 1.72E+06 and 4.37E+07 cfu/cm^2) than to Caco-2 cells, which simulates gum tissue (values between 1.71E+04 and 1E+06 cfu/cm^2), the differences being statistically significant ($P < 0.001$).

3.3. *Genetic bacterial identification and safety*

16S gene sequences confirmed that the seven selected strains belong to species with QPS status (Qualified Presumption of Safety) by EFSA (Table 4) based on their long history of apparent safe use. Moreover, following international guidelines for developing probiotics, in order to discard the presence of transferable antibiotic resistance genes in any of the candidate probiotic strains, antibiotic resistance profile was assessed. According to the breakpoints levels established by EFSA, all the strains were susceptible to all the antimicrobials tested (data not shown), corroborating their safety as probiotic strains.

In order to discard high lactic acid producing strains, the acidification due to the production of lactic acid by the candidate strains in different culture media resembling human diet was assessed. The pH values varied between 3.5 and 6.5 whilst $\log_{cfu/mL}$ values were between 8 and 9.5, showing that the strains had similar growth rates in the study conditions and therefore, the differences found were mainly attributed to the ability to produce acid. An acidification value by multiplying pH by $\log_{cfu/mL}$ was calculated, the greater this value is the less acid is produced. The ability to produce acid depends on the strain and the culture media. On average, production of acid values of pre-selected strains ranged between 31.92 and 53.26 (Table 4). Interestingly, oral isolated strains seem to produce less acid than those isolated from faeces (40.63 and 33.64 of median value, respectively). In general, the strains produced more acid when growing in the presence of glucose, fructose and lactose and less when the culture medium is supplemented with yeast extract or sucrose (Table 4).

4. Discussion

Control of dental plaque-related diseases (caries, gingivitis and periodontitis) has traditionally relied on non-specific removal of plaque by mechanical means. However, this tendency is changing towards more targeted treatments. Novel treatment approaches are focused on inhibiting specific small groups of organisms, single species or even key virulence factors they produce. Antibiotics were extensively used in the past but increasing problems of resistance to these agents have encouraged the search for alternative products or strategies. The use of plant-derived substances with antimicrobial activity,[38] functional inhibition approaches – including the use of protease inhibitors – or the replacement therapy by which a resident pathogen is replaced with a non-pathogenic microorganism are some of the treatments proposed to combat oral diseases.[39] Moreover, recently the use of probiotics for improving oral health has been proposed. Replacement and probiotic therapies have a major advantage when comparing with antimicrobial or functional inhibition approaches which is that besides their antimicrobial activity they have the ability to preclude the recolonization of the same pathogens.

The selection of the best probiotic strains for preventing periodontal diseases and halitosis is still an open issue. It seems necessary to perform specific screenings for selecting appropriate strains. In addition, to claim that a bacterial strain is a probiotic strain, several guidelines suggested by a joint

Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) working group must be followed.[40] These guidelines emphasize the necessity of correct identification of a probiotic strain and the use of various *in vitro* tests to evaluate its functionality and safety. It seems, therefore, necessary to identify all the critical qualities that could affect the efficacy of a probiotic for improving oral health and design *in vitro* tests for evaluating each one of these characteristics. As mentioned above, the objective of a probiotic therapy is the substitution of resident harmful pathogens for other non-pathogenic bacteria. For that, it is necessary that the probiotic presents antagonism activity against the pathogens to be replaced, as well as a high ability to colonize the oral cavity. In this sense, it is essential that the probiotic candidates show capacity of adhesion to oral surfaces, capacity of growing forming biofilms and high survival at oral conditions. Moreover, it is also necessary to discard the candidate probiotics which produce malodour compounds once they colonize the oral cavity. On the other hand, the issue of safety of a probiotic is of special concern. In addition to demonstrate the non-toxicity of the probiotic candidate and its incapacity of spreading antibiotic-resistant genes, it is necessary to evaluate the potential for the probiotic to produce caries since it has been described that some strains of *Lactobacillus* can induce caries.[11,41]

In this work, a screening of lactic acid bacteria was performed in order to select good candidate probiotic strains for preventing oral disorders, such as gingivitis, periodontitis and halitosis. Human origin of strains was a prerequisite, since most current successful strains are indicated to be isolated from human.[42] It is expected that a probiotic strain can function better in a similar environment to where it was originally isolated from. In this sense, strains isolated from oral-cavity of health individuals are preferable since they may be used to the co-existence with oral pathogens and may have enough ability to adhere to oral cavity. However, since it is estimated that approximately 10^{11} bacteria are swallowed daily, it is plausible that salivary strains would be also isolated from faeces.[43]

One hundred new isolates from saliva and faeces of healthy children were included at the beginning of the screening. After biochemical characterization, 54 strains were discarded because they did not belong to any lactic acid bacteria genera. The remaining 46 strains belonged mostly to *Lactobacillus* genus but *Lactococcus*, *Pediococcus* and *Leuconostoc* genera were also represented. The 46 lactic acid bacteria strains were subjected to a variety of *in vitro* tests to show their functionality. Commercial strains *S. salivarius* K12 and *L. reuteri* Prodentis were also included in order to elucidate if the new strains isolated in this work could be more effective on improving oral health than currently commercialized products.

Most of the new isolated strains seem to be resistant to oral conditions tested. It is interesting to note that the concentrations used in this work were between 67 and 320-fold higher than the physiological conditions, in order to select the most resistant strains. Eleven of the new strains showed better resistance to oral conditions than commercial *S. salivarius* K12 strain. Moreover, 45 out of the 46 new strains showed better survival ability than *L. reuteri* Prodentis, which reduces caries and gum diseases when it is consumed regularly.[27] In addition, ten and 38 of the new isolates showed greater aggregation activity than commercial control *S. salivarius* K12 and *L. reuteri*, respectively. This means that they showed greater capacity of establishing biofilms and inhibiting plaque formation by pathogens than *S. salivarius* K12 and *L. reuteri* Prodentis. The new isolated strains showed high antagonistic activity against oral pathogens, but whilst 11 strains showed activity against a unique specific pathogen, 11 were able to antagonize a wide number of bacteria. These results seem to indicate that two mechanisms, a specific inhibitory effect against particular oral pathogens and a general mechanism of inhibition, are present depending on the candidate probiotic strain. The strains with a broad inhibitory activity are the most interesting ones from an antagonistic activity point of view, especially if they inhibit *P. gingivalis*. Twenty-three out of the 46 strains showed better antagonistic activity – taking into account both the intensity of the inhibition and the number of pathogens antagonized – than *S. salivarius* K12, which was the best control strain, and *L. reuteri* Prodentis was amongst the worst strains according to their ability to antagonize oral pathogens. None of the strains produced unpleasant volatile compounds. In fact, only 6 out of the 46 strains showed a higher value than the control *L. reuteri*.

When comparing the results obtained by each strain in the different *in vitro* tests, as expected, no strain showed the best behaviour in all the tests performed. Therefore, to select the best candidate strains it is essential to prioritize the test which can be most informative. In order to establish an objective criterion of selection, searches in PubMed database were performed using different combinations of keywords as indicator of the magnitude of the relationship between the words analysed. For that, ''periodontitis'', ''gingivitis'' and ''halitosis'' terms were combined with each one of the test performed, ''lysozyme'', ''hydrogen peroxide'', ''aggregation'', ''production of malodour compounds'', ''oral pathogen'', ''*P. gingivalis*'' ''*F. nucleatum*'', ''*T. denticola*'', ''*P. denticola*'' and ''*S. mutans*'' and the number of publications were recorded (data not shown). The results obtained in these searches point out that antagonistic activity and aggregation ability are the most relevant properties related to these oral disorders. Following this criterion, the strains which showed most promising results were pre-selected. It is surprising that four out of the seven strains selected were isolated from human faeces. It is likely that those strains are swallowed oral cavity residents. Accordingly, the four faeces-isolated strains showed better adherence to pig tongue than oral cavity-isolated ones and the commercial control *S. salivarius* K12, suggesting that they must be used to being in contact with oral tissues. All the pre-selected strains showed better capacity of adhesion to pig tongue than *S. salivarius* K12, whilst three out of seven strains have greater adhesion to Caco-2 cells than the commercial strain.

The final steps of the screening process were aimed to assess the safety of the best candidate strains. In addition to their long history of apparent safe use (QPS status), the strains were susceptible to a battery of antibiotics according to EFSA guidelines, which is a prerequisite for a strain to be used as probiotic. On the other hand, it is described that one possible side-effect of applying a lactic acid bacterium for improving oral health is an increased risk of caries due to the production of lactic acid, and the resulting reduction of pH, as a result of fermentation of sugars present in human diet. Therefore, it is

desirable to use lactic acid bacteria with reduced ability to produce lactic acid. The comparison of the global value of acid production obtained by each strain revealed that all the strains had similar or lower acid production ability than *L. reuteri* Prodentis, which has not reported side-effects when administered to improving oral diseases.[27,28]

In conclusion, in this work lactic acid bacteria which possess good functional probiotic properties, such as antimicrobial activity against oral pathogens, ability to aggregate and to adhere to oral tissues or high tolerance to oral environmental stress factors were isolated and characterized. Moreover, in addition to have the QPS status by the EFSA, any safety risk assessed by the antibiotic resistance profile as well as the ability to produce lactic acid was discarded. Taking all these results together, it is suggested that at least seven of the new isolated lactic acid bacteria strains show promising properties to be used as potential probiotics, alone or as a part of a probiotic formula, for improving oral health. However, in order to demonstrate their efficacy for preventing oral diseases, controlled and well-designed clinical trials must be performed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.archoralbio.2011.10.006.

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