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Innovation in Restorative Dentistry

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Abstract

Oral infectious diseases are probably the most common infectious pathologies affecting humankind. They have a significant impact on both people quality of life and costs for the healthcare system. Thus, their prevention should be high priority for governaments and research institutes.

There are hundres of bacterial species in the oral environment, however, only a few of them are pathogenic. In fact, the disease begins when there is an imbalance in the oral biofilm with a prevalence of pathogenic species.

In order to prevent oral infectious diseases we can eliminate most of the oral biofilm with mechanical and chemical means. However, in this way also many saprophytic species are eradicated. A smart solution could be to induce a selective pressure for “good” oral bacteria thus hampering pathogenic ones. How can we achive this? Modifying our diet is a possible solution, we demonstrated how cariogenic biofilm development was lowered by using levorotatory carbohydrates instead of dextrorotatory ones.

If we already have a carious lesion, it should be removed and the tooth restored with proper materials. However, most of them are methacrylate based and favour biofilm development, moreover, they also seem to select cariogenic species due to the lack of buffering ability. In our study we demonstrated how the use of different materials, the siloranes, could lead to a decrease in the biofilm development, thus theoretically lowering the incidence of secondary

caries.

If a tooth cannot be recovered and should be extracted, dental implants are probably the best solution for their replacement. However, peri-implantitis is a serious issue affecting up to 50% of the implant and can lead to their loss. The prevention of this oral disease is hence very important. In our study we compared different materials and showed that biofilm formation was similar in all of them. In future studies we will investigate if the biofilm on these materials is similar or not and if they are prevalently pathogenic or saprophytic ones.

In conclusion, oral infectious disease are still very common and for decades dentists tried to achieve oral health by eliminating all the biofilm. However, the most innovative strategy is not to eradicate it but to induce selective pressures by using different means thus leading to a beneficial biofilm which does not cause illness but instead promote our health.

Sommario

Le malattie infettive del cavo orale sono tra le più diffuse nel genere umano. Queste hanno un grande impatto sulla qualità della vita delle persone e il costo per il loro trattamento risulta essere molto elevato, sia per i cittadini che per i sistemi sanitari nazionali. La prevenzione di queste patologie dovrebbe quindi essere altamente prioritaria.

Causa di queste malattie sono quindi i batteri, le specie che popolano il cavo orale risultano essere diverse centinaia ma solo una ristretta minoranza è in grado di indurre patologie. La malattia comincia infatti quando nel biofilm orale le specie patogeniche diventano prevalenti rispetto a quelle saprofite, parliamo quindi di biofilm disbiotici.

Da sempre, la soluzione adottata per prevenire le patologie infettive del cavo orale come carie e parodontite è quella di andare a rimuovere meccanicamente il biofilm attraverso lo spazzolamento degli elementi dentari. In aiuto alle manovre meccaniche si utilizzano talvolta anche mezzi chimici come collutori a base di clorexidina, un potente disinfettante. Queste metodiche non sono però selettive sulle specie microbiche pericolose per la salute ma vanno a eradicare indistintamente sia microorganismi patogenici che saprofiti. Oltretutto, una buona parte delle persone non è in grado di raggiungere un'igiene orale ottimale. La soluzione più intelligente potrebbe essere quella di indurre delle

pressioni selettive per i batteri saprofiti, sfavorendo quindi quelli patogenici. Per raggiungere questo obiettivo possiamo agire in modi diversi. Ad esempio modificando la dieta, uno studio condotto per questa tesi ha infatti dimostrato come lo sviluppo del biofilm cariogenico sia stato rallentato notevolmente usando carboidrati levogiri rispetto ai più comuni destrogiri.

Qualora non sia stato possibile prevenire le lesioni cariose e i denti necessitino di essere curati, i materiali più usati per ricostruire i tessuti dentari andati persi sono i compositi a base metacrilica. Purtroppo però questi materiali accumulano più biofilm rispetto ai denti sani e sembra che possano anche esercitare una pressione selettiva sulle specie patogeniche in quanto privi di azione tampone nei confronti del pH. In questa tesi siamo andati ad osservare come l'uso di materiali compositi a base siloranica risultino essere meno soggetti alla formazione di biofilm a parità di tempo di polimerizzazione rispetto a quelli metacrilici.

Infine, quando un elemento non può più essere recuperato e necessita di essere estratto, gli impianti risultano essere spesso la miglior terapia per sostituirli. Tuttavia questi risultano essere soggetti a peri-implantite con percentuali che arrivano anche al 50%. La prevenzione di quest'ultimo risulta fondamentale per la prognosi a lungo termine degli impianti. Nella tesi abbiamo studiato lo sviluppo di biofilm su diversi materiali senza trovare differenze. Studi futuri andranno ad esaminare se questi biofilm siano simili dal punto di vista qualitativo e se siano composti da specie prevalentemente patogeniche o

saprofite.

In conclusione, le malattie infettive del cavo orale sono ancora molto comuni e ad oggi la soluzione più comunemente adottata dai dentisti è stata quella di eliminare tutto il biofilm. Tuttavia, la soluzione più innovativa potrebbe essere quella di produrre delle pressioni selettive, usando differenti approcci, con lo scopo di avere un biofilm equilibrato, in grado di promuovere esso stesso la nostra salute invece di indurre malattia.

1 Introduction

1.1 Innovation

According to the Oxford Dictionary of English innovation is: “a new method, idea, product, etc.” (Stevenson, 2010) It can also be defined as something more effective when compared to previously developed objects, ideas or protocols. Innovation can be both “disruptive” if it leads to the redefinition of a procedure or “sustaining” if it allows do to something better than before. (Chambers, 2001) Dentists are generally great innovators due to their ability to find a use in their profession for many already existing technologies with the main objective of improving the quality of their works. PhDs are all about innovation, both disruptive and sustaining. So, the philosophy behind this thesis was to go a step forward in bridging the gap between research and clinics. In particular, two aspects were investigated: caries prevention and materials development.

1.2 Innovation in prevention

1.2.1 The importance of prevention

Dental diseases have almost non-existant mortality rate in developed countries but they have a very high impact on the quality of life of the

affected people. (Moynihan & Petersen, 2004) In fact, teeth are needed for a correct nutrition, fonation and integration into society. (Moynihan & Petersen, 2004)

In the US, according to CDC, dental caries affects about 25% of children aged between two and five and about 50% of those aged between twelve and fifteen.(Dye et al., 2007)

Dental caries are very expensive for health care services. In most of the developed countries up to 10% of health care expenses are accounted for their treatment. (Sheiham, 2001) This is due to the fact that fillings, root canal therapies, extractions, implants are very expensive, expecially if compared with preventive strategies. Moreover, restored teeth have an increased risk of future disease thus leading to other more expensive treatments.

It can be concluded that prevention should be a priority both for improving patients quality of life and reduce health care expenses.

1.2.2 What is dental caries?

The term “dental caries” is used to identify two correlated aspects:

-The carious process

-The signs of this process

The carious process is caused by the metabolic activity of the bacteria inside oral biofilm, which cannot be eradicated and hence is always present. (Kidd, 2011) However this process does not always end up in a cavitated lesion of the dental hard tissues. In fact, the formation of carious lesions depends on the composition of the oral biofilm. If cariogenic bacteria become predominant in the oral biofilm, it is more likely for carious lesions to appear. (Fejerskov, Kidd, Nyvad, & Bælum, 2008)

1.2.3 Perimplantitis

Dental caries and periodontal disease can lead to the impossibility of mantaining some teeth, hence surgical extraction is necessary. Nowdays, in order to replace missing teeth, dental implants are probably the best solution since they usually represent the least invasive prodcedure and do not need, sound tissue removal from adjacent teeth to create a traditional fixed prosthesis. However, there are some issues with dental implants, since,

although being a very succesful treatment mode, they are subjected to peri-implantitis, even after many years of osseointegration. (Atieh, Alsabeeha, Faggion, & Duncan, 2013) Peri-implantitis is a localized infectious disease that generates an inflammatory process which leads to the loss of bone around dental implants. (Mombelli & Lang, 1998) The prevalence of this disease ranges between 7.8% and 43.3% (Konstantinidis, Kotsakis, Gerdes, & Walter, 2015; Mir-Mari, Mir-Orfila, Figueiredo, Valmaseda-Castellon, & Gay-Escoda, 2012) often leading to the implant loss. Being an infectious disease, the presence of bacteria on the implant is required for the inflammatory response to begin. Thus, strategies to prevent bacteria adhesion and subsequent biofilm formation could be useful in saving many implants from failure. Among these strategies, the use of different materials for implants could help in eliminating or at least reducing biofilm formation. As for dental caries, the biofilm is not pathogenic per se, it dipendes on the prevalence of different species inside. It should be noted that a different material for implantology could lead to similar biofilm development but with less pathogenic species or, on the opposite, more of them. In this PhD thesis the influence of Polyether-ether-ketone (PEEK) (Fuhrmann, Steiner, Freitag-Wolf, & Kern, 2014; Wang et al., 2015) was evaluated since it seems to be a promising material for both orthopedic and dental implants.

1.2.4 Disbiosis: imbalance of dental plaque

Hundreds of different microbial species are present in dental plaque. (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005) Dental plaque is a very complex biofilm, which is defined as a group of microorganisms in which they adhere to each other and are embedded in a self-made extracellular matrix composed by extracellular polymeric substances (EPS) composed by polysaccharides, proteins, lipids and also nucleic acids. (Zijnge et al., 2010) The oral cavity is an hostile enviroment and thus, the ability of micro-organisms to surive heavily depends on the capacity to form biofilms which are up to 1000 times more resistant to antimicrobial agents than planktonic bacteria. (Jenkinson & Lamont, 2005) The first step in the formation of a biofilm on teeth surfaces is bacterial adhesion. Bacteria does not adhere directly to enamel and dentin, they adhere to a salivary protein-based layer called the acquired pellicle. (Marsh & Bradshaw, 1995) Hence, the interactions between hard tissues, bacteria and oral fluids are all negotiated by this pellicle which can totally or partially mask them, depending on its thickness. (C. Hannig & Hannig, 2009) On the other hand microbial adhesion is also dependant on long-range forces which are transferred by the pellicle. (C. Hannig & Hannig, 2009) However it has to be clear that it also has useful purposes, in fact, due to its salivary origin, it is composed by antibacterial agents such as lysozime and immunoglobulins. (M. Hannig & Joiner, 2006) Other than that, the pellicle also mediates all the

mineralization/demineralization processes. After bacterial adhesion, the biofilm begins to form with the production of EPS matrix and the cellular division of the early colonizers. (Kolenbrander et al., 2006) It has to be noted that most of the mass of the dental plaque is formed by cellular replication rather than aggregation of new bacteria from the external enviroment. (Kolenbrander et al., 2006)

According to the currently accepted ecological plaque hypothesis, it is now commonly acknowledged that oral biofilm is not pathogenic by itself, only a small fraction of the hundreds of species cause dental diseases. (Marsh, 1991)

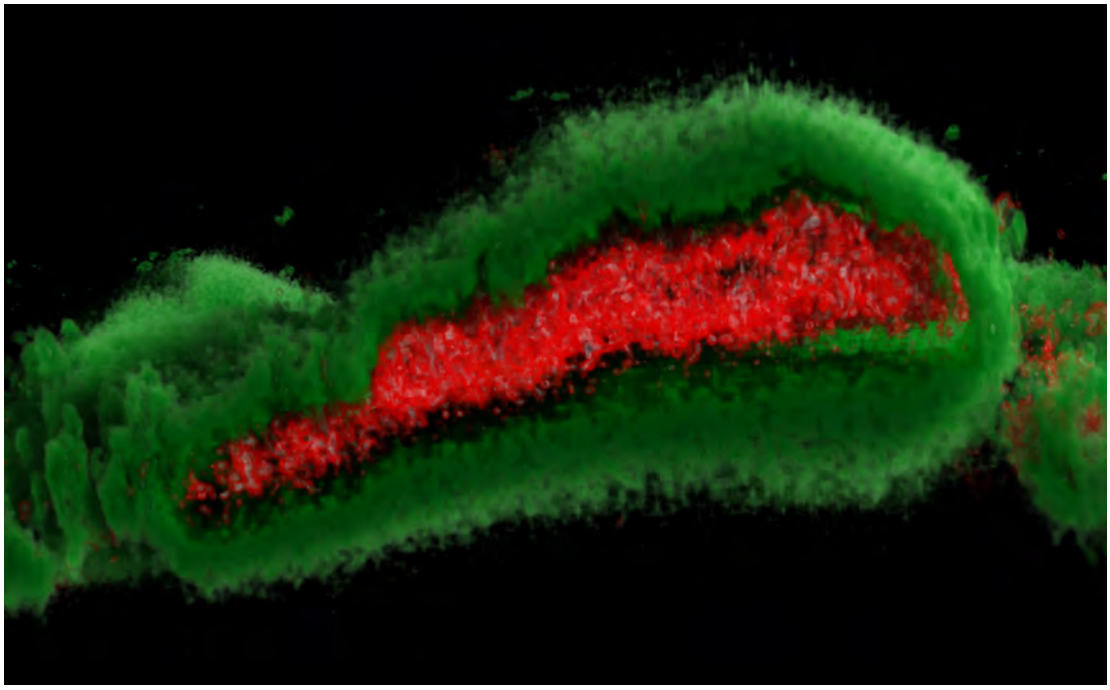
In healthy conditions there is a balance between the biofilm and the host. However, several factors can cause the disruption of this equilibrium, called dysbiosis, (Pflughoeft & Versalovic, 2012) thus leading to the prevalence of pathogenic species with the subsequent onset of oral diseases such as dental caries and periodontal disease.

The main aetiological agent of dental caries is considered to be *S. mutans*(*Metwalli, Khan, Krom, & Jabra-Rizk, 2013*) **(Fig. 1)** but there are also many different microorganisms related to this disease such as many other streptococcal species like *S. sobrinus* and also *Lactobacillus* spp. (Hardie, 1992)

Dentists generally treat dental caries with the removal of the affected tissue and the subsequent reconstruction. However, resin based composites (RBCs), generally used for fillings were proved to be more prone towards biofilm formation both *in vivo* and *in vitro* when compared to natural tissues. *(Hahn, Weiger, Netuschil, & Bruch, 1993)* Also, on these materials there is a lack of buffering system by the dissolution of calcium ions present in the mineral matrix of the enamel and dentin. This can lead to an increased demineralization of the surrounding tissues and furthermore, acidic conditions can select acid-resistant species such as *S. mutans, Lactobacillus* spp. which furtherly increase caries risk. (Thomas, van der Mei, van der Veen, de Soet, & Huysmans, 2008)

To conclude, many attemps were made in the development of materials less prone to biofilm formation, expecially a pathogenic one. On the other hand, since there are no serious alternatives to RBCs materials for fillings, patients should always be educated in oral hygiene and on a correct diets, including foods able to promote the formation of balanced biofilm.

Fig. 1
3D reconstruction from Confocal Laser Scanning Microscopy images.
Micro-colony in a biofilm of *S. mutans*.
Live/ Dead stain was applied (Syto9/propidium iodide). Live bacteria exhibited green fluorescence, and bacteria with compromised membranes exhibited red fluorescence.



2 Experiments

2.1 Innovation in caries prevention

2.2 Introduction

2.2.1 The power of carbohydrates: innovative approach to restore the balance without necessary killing bacteria

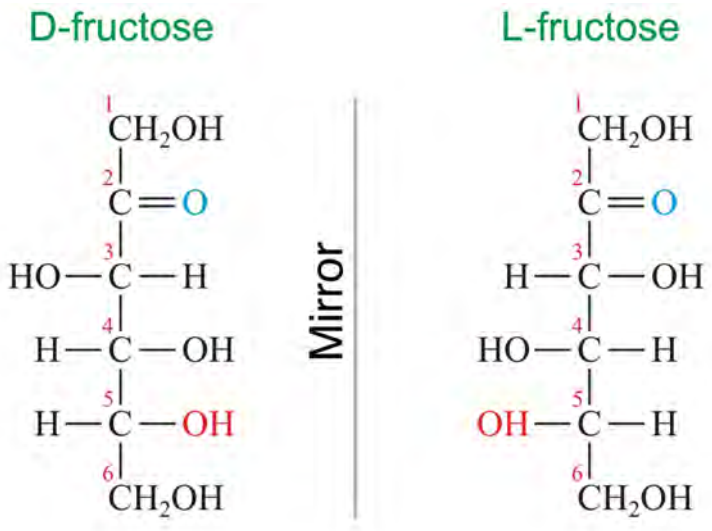
It is well known that bacteria use carbohydrates as a primary energy source and to develop the structural elements of the extracellular matrix. (Marsh, 2006) Interestingly, some carbohydrates are known to interfere with microbial adhesion and biofilm formation. In particular, levorotatory carbohydrates (L-carbohydrates) **(Fig. 2)** are metabolized to a significantly lesser degree by microbial enzymatic systems than the corresponding dextrorotatory forms (D-carbohydrates).(Livesey & Brown, 1995; Moazeni, Zhang, & Sun, 2010) Moreover, some polyols cannot be metabolized by most of dental plaque microorganisms.(Goncalves et al., 2006; Maguire, Rugg-Gunn, & Wright, 2000) In particular, several studies showed the efficacy of xylitol in reducing bacterial adhesion and biofilm formation *in vitro*, *in situ*, and *in vivo.(Burt, 2006; ElSalhy, Sayed Zahid, & Honkala, 2012; Lee, Choi, & Kim, 2012; Marttinen et al., 2012)* It is known that *S. mutans* is very versatile in promptly metabolizing a series of different sugars(Colby & Russell, 1997) and possesses inducible enzymes which

allow it to metabolize polyols, such as sorbitol and mannitol.(Brown & Wittenberger, 1973)

2.2.2 Levorotatory carbohydrates and xylitol subdue Streptococcus mutans and Candida albicans adhesion and biofilm formation

As already stated, dietary carbohydrates and polyols affect the microbial colonization of oral surfaces by modulating adhesion and biofilm formation. However, no study has evaluated *S. mutans* behavior in presence of L-carbohydrates. Furthermore, very few studies have thus far evaluated the effect of xylitol and other polyols during *C. albicans* biofilm formation (Ichikawa, Yano, Fujita, Kashiwabara, & Nagao, 2008) and no study has evaluated its behavior when in presence of L-carbohydrates.

Fig. 2
Example of dextro and levorotatory form of a fructose.
Levorotatory carbohydrates are generally less prone to be metabolized by bacteria.



2.2.3 Aim of the study

The aim of the present study was to evaluate the effect of a group of L-carbohydrates and their dextrorotatory counterparts, as well as three polyols (sorbitol, mannitol, and xylitol) on *in vitro S. mutans* or *C. albicans* adhesion and biofilm formation. The null hypothesis was that L-carbohydrates and the polyols derived from the tested carbohydrates do not significantly reduce *in vitro S. mutans* or *C. albicans* adhesion and biofilm formation on polystyrene surfaces when compared with the corresponding D-carbohydrates.

2.2.4 Materials and Methods

Microorganisms

All the culture media were obtained from Becton–Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA).

A pure suspension of *S. mutans* strain ATCC 35668 in brain–heart infusion broth (BHI) was obtained after a 12 h incubation at 37 °C in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (1500 g at 19 °C for 5 min), washed twice with sterile phosphate-buffered saline (PBS), and resuspended in the same buffer. The cell suspension was subsequently subjected to sonication (Sonifier model B-150; Branson, Danbury, CT, USA; operating at 7W energy output for 30s) in order to disperse bacterial chains, then the suspension adjusted to a turbidity equivalent to that of a 1.0 McFarland

standard.

A pure suspension of *C. albicans* strain ATCC 90028 in BHI was obtained after a 24 h incubation at 37 °C in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (1500 g at 19 °C for 5 min), washed twice with sterile PBS and resuspended in the same buffer, and the suspension adjusted to a turbidity equivalent to that of a 1.0 McFarland standard.

Sugars

All reagents, including the different tested sugars and the multi-well plates used were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

The enantiomers of the following sugars were individually evaluated for adhesion and biofilm formation of the organisms: D- and L-glucose, D- and L-mannose, as well as enantiomer pair combinations in raceme solutions (50% D-glucose and 50% L-glucose; 50% D-mannose and 50% L-mannose; additionally, a 50% L-glucose and a 50% L-mannose solution was also tested. Furthermore, we included the following three polyols in the study (two of which corresponded to glucose and mannose): sorbitol, mannitol, and xylitol.

In order to ascertain the optimal concentration of sugar that exhibit the greatest biofilm activity, a preliminary test was performed with 1.25, 2.5, 5, and 10% wt./vol solution of D- and L-sugars diluted in Trypticase-Soy broth (TSB). TSB was chosen for its relatively low-sugar content (0.25% wt./vol glucose). A pure

TSB solution (30 g/L) with no extra sugar addition was used as negative control.

All the solutions used in the experiments were sterilized at 121 °C for 15 min, then stored at 4 °C until use.

Cell adhesion and biofilm formation

Three independent experimental runs were performed in three different weeks in order to exclude day-to-day variability, and data from the three runs were averaged.

A total of 180 ml of each of the tested sugar solutions and 20 ml of the standardized inoculum were inoculated into each well of 96-well plates; 24 replicate wells were inoculated in tandem for each sugar solution and strain. The plates were incubated at 37°C and 5% CO_2 supplemented atmosphere. The cell adhesion to the tissue-culture-treated polystyrene surfaces (inside the wells) was assessed for both tested strains using the MTT assay on 12 of the replicate wells after 2 h of incubation. The biofilm formation on the polystyrene surfaces was evaluated for the two tested strains using the MTT assay on the remaining 12 replicate wells after 24 h of incubation.

As *C. albicans* is invasive when it produces hyphae, and TSB is a complex medium which does not stimulate hyphae formation by *C. albicans*, a further experiment was performed diluting the tested sugar solutions into Lee's medium depleted of the original 1.25% wt. D-glucose content. *C. albicans*

biofilm formation on the polystyrene surfaces was subsequently evaluated using the MTT assay on additional 12 replicate wells after 24 h of incubation as previously specified.

MTT Assay

MTT assay was conducted as previously described.(Brambilla et al., 2014) Briefly, two starter MTT stock solutions were prepared by dissolving 5 mg/ml 3-(4,5)-dimethylthiazol-2-yl-2,5- diphenyltetrazolium bromide in sterile PBS, and 0.3 mg/ml of N-methylphenazinium methyl sulphate (PMS) in sterile PBS. The solutions were stored at 2 °C in light-proof vials until the day of the experiment, when a fresh measurement solution (FMS) was made by mixing 1 ml of MTT stock solution, 1 ml of PMS stock solution and 8ml of sterile PBS. A lysing solution (LS) was prepared by dissolving 10% v/v of sodium dodecyl sulphate and 50% v/v of dimethylformamide in distilled water.

At the end of the incubation period, the suspension was removed from the wells by gentle aspiration. The wells were then carefully washed three times with sterile PBS in order to remove non-adherent cells. After that, the plates underwent MTT assay for the evaluation of adherent, viable, and metabolically active biomass as follows: 100 ml of FMS were pipetted into each well and the plates were incubated for 3 h at 37 °C in light-proof conditions. During incubation, electron transport across the microbial plasma membrane and, to a lesser extent, microbial redox systems converted the yellow MTT salt to

insoluble purple formazan. The conversion was facilitated by the intermediate electron acceptor (PMS). The unreacted FMS was gently removed from the wells by aspiration and the formazan crystals were then dissolved by adding 100 ml of LS into each well and further incubated for 1 h at room temperature in light-proof conditions. About 90 ml of the suspension were then removed from each well and optical density (at 550 nm) was measured with a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA).

Scanning electron microscopy (SEM) evaluation

Round polystyrene specimens (6.4 mm diameter) were prepared from the bottom of 48-well tissue-culture-treated plates by means of a flame-heated custom-made circular punch. The specimens were inserted into new 48-well plates (one specimen per well) and then sterilized with a chemical peroxide-ion plasma sterilizer (STERRAD, ASP, Irvine, CA, USA) for 60 min at a maximum temperature of 45 °C to prevent heat-induced modification of the specimen surfaces.

After that, 360 ml of sugar solution and 40 ml of cell suspension from each of the tested strains were inoculated into each well of the sterilized plates. Four specimens were used for each sugar solution and strain. The plates were then incubated at 37°C and 5% CO_2 supplemented atmosphere for either 2 h (cell adhesion evaluation, n = 2 specimens/group) or 24 h (biofilm formation, n = 2 specimens/group). Additionally, two specimens per group were incubated with

C. albicans and the tested solutions prepared using glucose-depleted Lee's medium for 24 h.

Afterwards, each specimen was gently rinsed three times with sterile PBS to remove non-adherent cells and placed in a 2% glutaraldehyde–cacodylate-buffered (pH = 7.4) fixative solution for 48 h. The specimens were then passed through a graded ethanol series (50, 70, 80, 85, 90, 95, and 100%, v/v). Finally, the specimens were subjected to critical point drying (Critical-Point Dryer, EMS 850, Hatfield, PA, USA), mounted on stubs with conductive glue, sputter coated (JEOL FFC-1100, Japan), and observed with a scanning electron microscope (JEOL JSM-5300, Japan) at a magnification of 1000-4000x. Four randomly selected fields were recorded for each specimen.

Statistical analysis

Statistical analysis was performed using JMP 10.0 software (SAS Institute, Cary, NC, USA). Homogeneity of variances was preliminarily checked and verified using Bartlett's test. One-way ANOVA was employed to investigate differences in viable biomass OD values between the experimental groups, and Student–Newman–Keuls post-hoc paired test was employed to analyze significant differences whenever ANOVA yielded significant results. Two-way ANOVA model was used to investigate differences in *C. albicans* biofilm formation considering the two factors: growth medium (TSB vs. Lee's) and sugar solution. The level of significance (a) was set to 0.05.

2.2.5 Results

The preliminary testing of the different concentrations of the tested carbohydrate solutions indicated that as D-sugar concentrations increased, so did biofilm formation of both tested isolates, and a plateau value was reached for both isolates at 5% wt./vol. In **Table 1**, an example is given for D-glucose. L-sugars concentrations did not significantly alter biofilm formation of both the tested isolates at any concentration (**Table 1**, example for L-glucose). Therefore, a 5% wt./vol carbohydrates concentrations was used for all the sugar and polyol solutions and for both TSB and Lee's medium.

Table 1
S. mutans and *C. albicans* biofilm formation at 24 h at different glucose concentrations

	Biofilm formation (24h)	
	Streptococcus mutans	*Candida albicans*
	Mean (±1SD)	Mean (±1SD)
10% D-glucose	0.7493 (0.0628)[a]	1.0502 (0.1451)[a]
5% D-glucose	0.7024 (0.0763)[a]	0.9998 (0.1334)[a]
2.5% D-glucose	0.6059 (0.0807)[b]	0.6940 (0.1089)[b]
1.25% D-glucose	0.5414 (0.0992)[b,c]	0.5056 (0.0840)[c]
10% L-glucose	0.4470 (0.0744)[c,d]	0.1125 (0.0136)[d]
5% L-glucose	0.4310 (0.1493)[c,d]	0.1150 (0.0141)[d]
2.5% L-glucose	0.4347 (0.1093)[d]	0.1399 (0.0100)[d]
1.25% L-glucose	0.4817 (0.0736)[d]	0.1394 (0.0099)[d]
TSB (control)	0.4799 (0.0756)[d]	0.1598 (0.0263)[d]

Adhesion evaluation

The results for *S. mutans* and *C. albicans* adhesion to the polystyrene substrata after 2 h are shown in **Figs. 3** and **4**, respectively.

Fig. 3
S. mutans adhesion. The graph represents the amount of *S. mutans* viable biomass adherent to the polystyrene surfaces suspended in different TSB-diluted carbohydrates. Mean and standard error (SE) are indicated; bars with different superscript letters are significantly different ($p<0.05$)

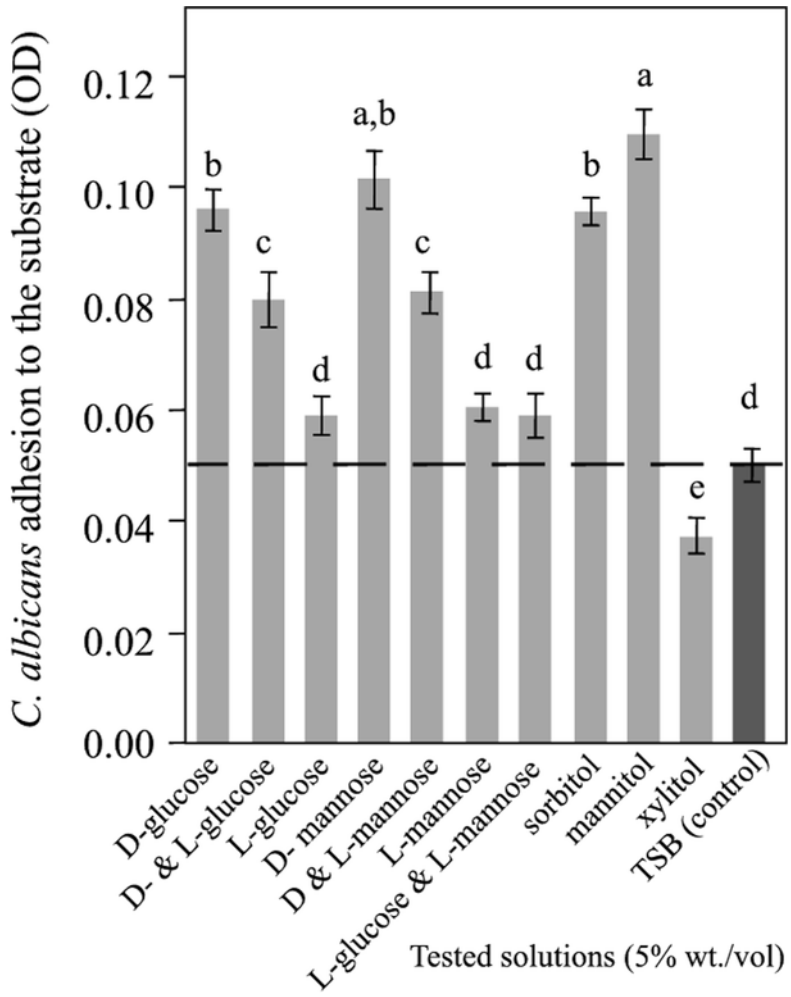
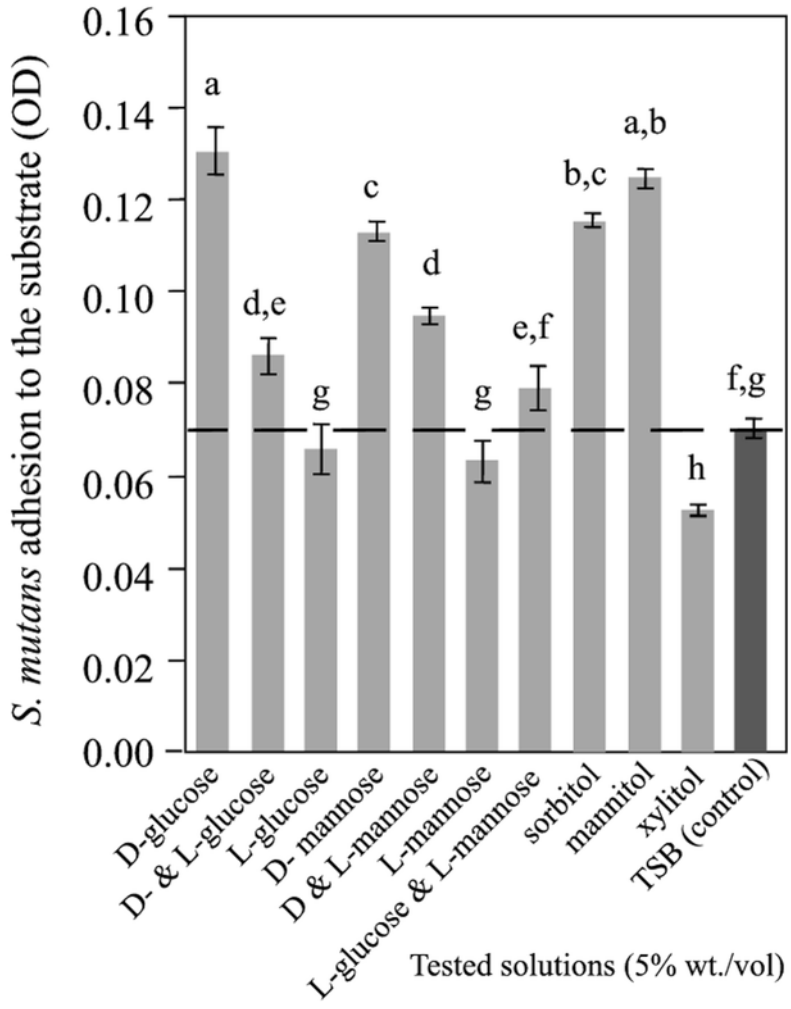


Fig. 4
C. albicans adhesion. The graph repreents the amount of *C. albicans* viable biomass adherent to the polystyrene surfaces suspended in different TSB-diluted carbohydrates. Mean and standard error (SE) are indicated; bars with different superscript letters are significantly different (p>0.05)



The percentage of variation of the different groups in comparison to the negative control group (TSB) as well as the statistical significativity (p-value) for each comparison is shown in **Table 2**.

Table 2
S. mutans and *C. albicans* adhesion and biofilm formation

	Adhesion (2h)			
	Streptococcus mutans		*Candida albicans*	
	% change to control	*p*-value	% change to control	*p*-value
TSB (control)				
D-glucose	+ 86%	< 0.0001*	+ 92%	< 0.0001*
D- & L-glucose	+ 22%	0.0019*	+ 59%	< 0.0001*
L-glucose	- 6%	0.3816	+ 18%	0.1058
D-mannose	+ 61%	< 0.0001*	+ 103%	< 0.0001*
D- and L-mannose	+ 35%	< 0.0001*	+ 62%	< 0.0001*
L-mannose	- 10%	0.1516	+ 21%	0.0537
L-glucose and L-mannose	+ 12%	0.0878	+ 18%	0.1061
Sorbitol	+ 64%	< 0.0001*	+ 91%	< 0.0001*
Mannitol	+ 78%	< 0.0001*	+ 119%	< 0.0001*
Xylitol	-25%	0.0006*	-25%	0.0216*
	Biofilm formation (24h)			
	Streptococcus mutans		*Candida albicans*	
	% change to control	*p*-value	% change to control	*p*-value
TSB (control)				
D-glucose	+ 40%	< 0.0001*	+ 530%	< 0.0001*
D- and L-glucose	+ 24%	0.0005*	+ 429%	< 0.0001*
L-glucose	- 3%	0.6737	- 3%	0.8902
D-mannose	+ 19%	0.0038*	+ 433%	< 0.0001*
D- and L-mannose	+ 29%	< 0.0001*	+ 404%	< 0.0001*
L-mannose	- 1%	0.8215	+ 22%	0.2783
L-glucose and L-mannose	+ 2%	0.7834	- 19%	0.3387
Sorbitol	+ 25%	0.0002*	+ 37%	0.0691
Mannitol	+ 18%	0.0075*	+ 30%	0.1317
Xylitol	+ 2%	0.7293	- 9%	0.6628

Influence of the different tested solutions on *S. mutans* and *C. albicans* adhesion and biofilm formation when compared to the negative control group (TSB). The significativity levels (p values) obtained from Student–Newman–Keuls post-hoc test are displayed. The symbol * highlights significant differences as found by the post-hoc test.

S. mutans and *C. albicans* adhesion in response to the tested solutions showed a similar general trend. Results displayed high *S. mutans* adhesion to the substrata in the presence of D-carbohydrates (+86% for D-glucose and +61% for D-mannose), intermediate values with the raceme solutions (+22% for D- and L-glucose and +35% for D- and L-mannose blends) and significantly lower adhesion in the presence of the L-forms (**Table 2**). Sorbitol and mannitol showed adhesion values similar to the corresponding D-carbohydrates with a +64 and +78% increase in adherent biomass, respectively. *C. albicans* adhesion

in response to the tested solutions displayed high-adhesion values in the presence of D-carbohydrates (+92% for D-glucose and +103% for D-mannose), intermediate values with the raceme solutions (+59% for D- and L-glucose and +62% for D- and L-mannose blends) and significantly lower adhesion in the presence of the L-forms (**Table 2**). Sorbitol and mannitol showed similar adhesion values to the corresponding D-carbohydrates, a +91 and +119% increase in adherent biomass, respectively. In both strains, xylitol elicited the lowest adhesion values, which were significantly lower (-25%, $p < 0.01$ for both strains) than those elicited by L-carbohydrates solutions or the negative controls.

Biofilm formation

The results for *S. mutans* and *C. albicans* biofilm formation after 24 h are shown in **Figs. 5** and **6**, respectively.

Fig. 5

S. mutans biofilm formation. The graph represents the amount of *S. mutans* viable biomass developed as biofilm on the polystyrene surfaces suspended in different TSB-diluted carbohydrates. Mean and standard error (SE) are indicated; bars with different superscript letters are significantly different ($p < 0.05$).

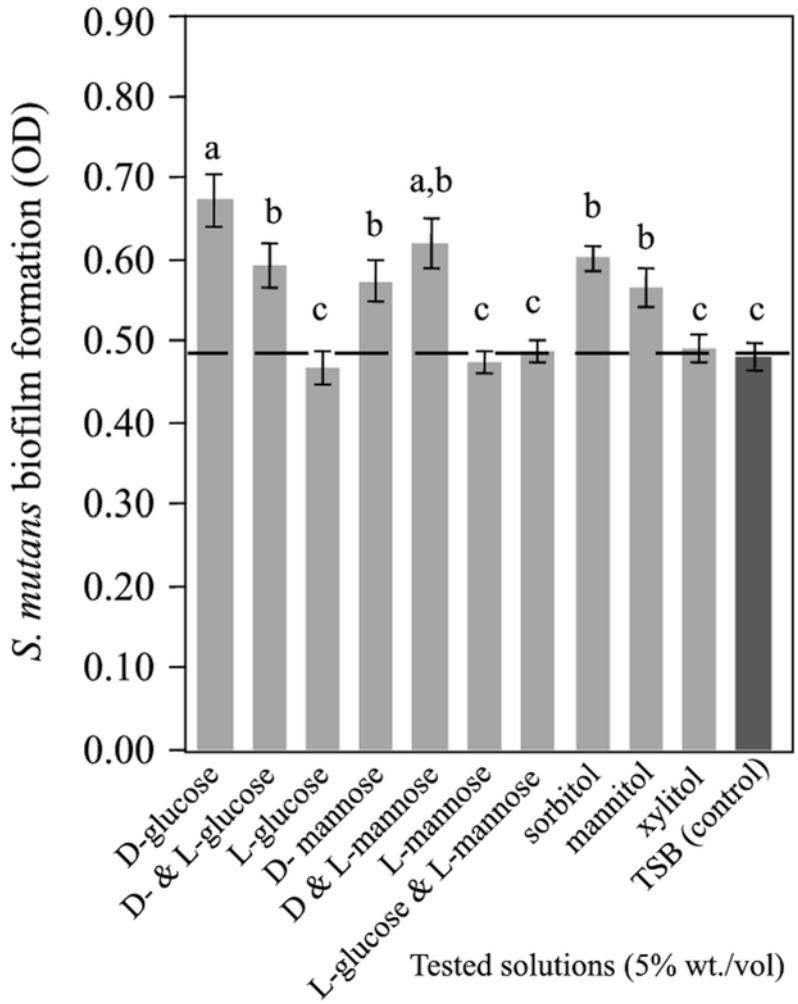
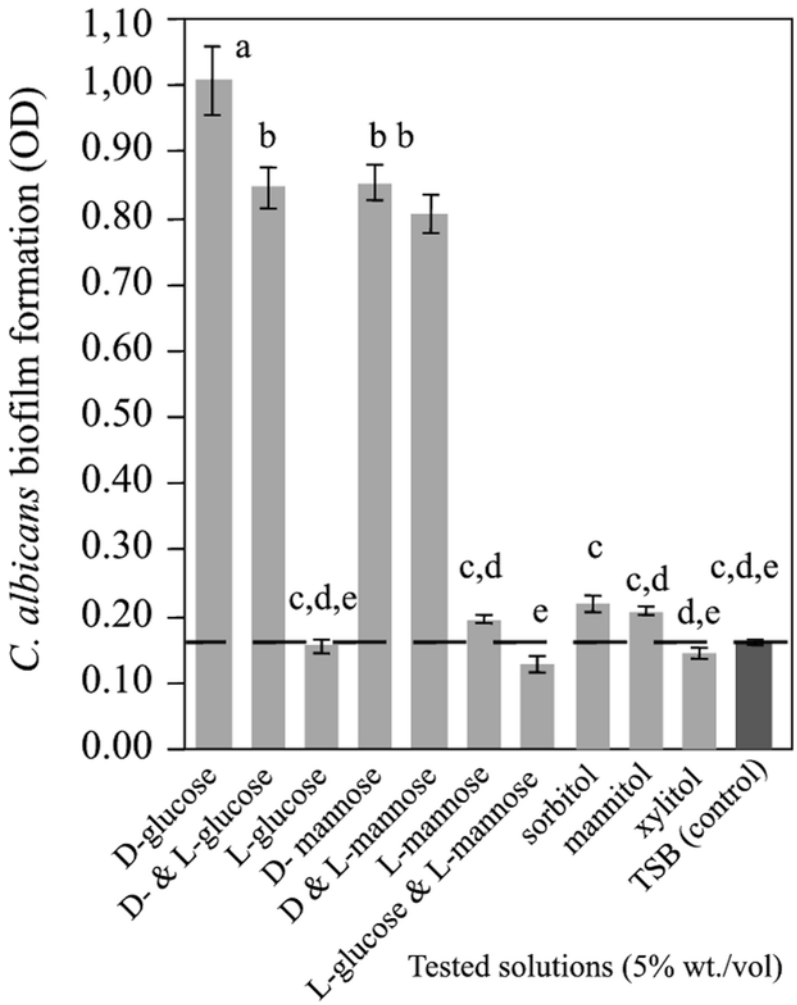


Fig. 6
C. albicans biofilm formation. The graph represents the amount of *C. albicans* viable biomass developed as biofilm on the polystyrene surfaces suspended in different TSB-diluted carbohydrates. Mean and standard error (SE) are indicated; bars with different superscript letters are significantly different ($p < 0.05$).



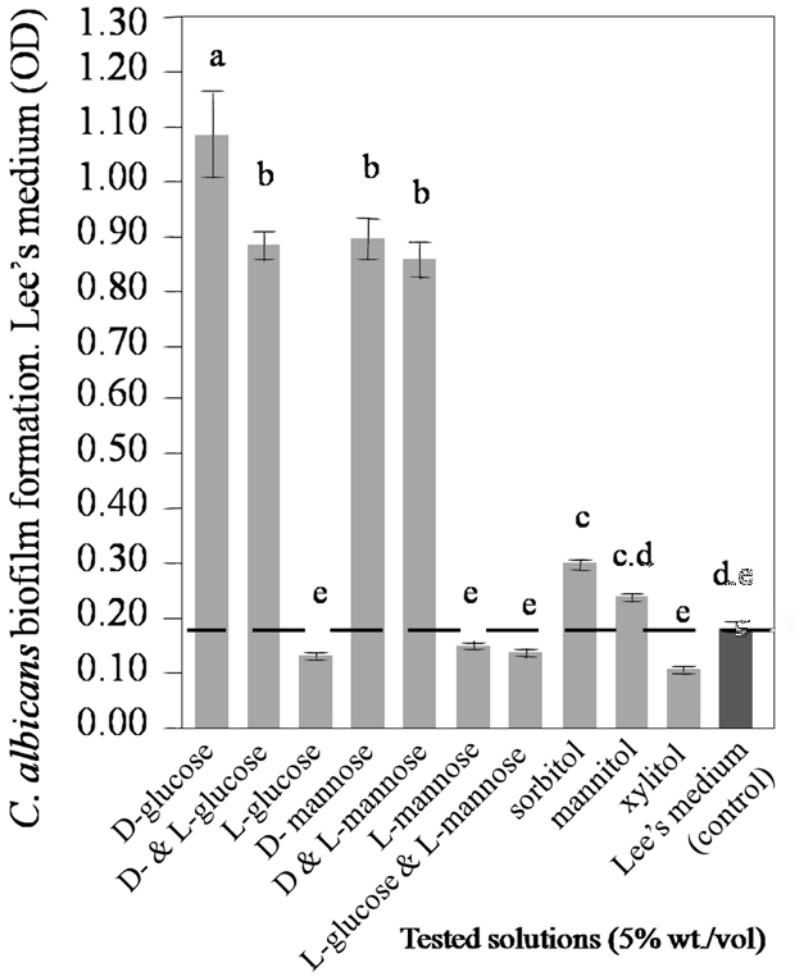
The percentage of variation of the different groups in comparison to the negative control group (TSB) as well as the statistical significance (p-value) for each comparison is shown in **Table 2**. As shown in **Fig. 5**, a significantly higher degree of *S. mutans* biofilm formation was noted in presence of D-glucose

(+40%), intermediate values with D-mannose (+19%) and the raceme solutions (+24% for the D- and L-glucose and +29% for the D- and L-mannose) and significantly lower biofilm development in the L-form solutions (-3% for L-glucose, -1% for L-mannose, and +2% for L-glucose and -mannose, all similar to the control). No significant differences in biofilm formation were noted between sorbitol, mannitol (+25% and +18%, respectively) and the corresponding D-carbohydrates and raceme solutions, except D-glucose ($p < 0.05$). The degree of *S. mutans* biofilm formation elicited by xylitol was similar to those of L-carbohydrates and the negative control). The overall trend of *C. albicans* biofilm formation in the solutions of the tested carbohydrates was in general similar to that of *S. mutans* in that the D-forms elicited profuse biofilm growth and the L-forms, moderate to scanty growth

(**Fig. 6**). Nevertheless, on naked eye observation it appeared that the L-carbohydrates and the polyols elicited a much lower degree of biofilm formation than the D-forms (+530% for D-glucose and +433% for D-mannose) and the raceme solutions (+429% for D- and L-glucose and +404% for D- and L-mannose).

Data for *C. albicans* biofilm formation in glucose-depleted Lee's medium is shown in **Fig. 7**.

Fig. 7
C. albicans biofilm formation. The graph represents the amount of *C. albicans* viable biomass developed as biofilm on the polystyrene surfaces suspended in different glucose-depleted Lee's medium-diluted carbohydrates. Mean and standard error (SE) are indicated; bars with different superscript letters are significantly different ($p < 0.05$)



Two-way ANOVA did not find significant interactions between the considered factors ($p = 0.2784$). A high-statistical significance was found for the factor sugar solutions ($p < 0.0001$) but no significance was found for the factor growth medium ($p = 0.0621$). *C. albicans* biofilm formation in response to the different solutions obtained using Lee's medium showed the same trend as with TSB

growth medium, with the exception of sorbitol who promoted a significantly higher biofilm formation than the control medium ($p = 0.0072$).

SEM evaluation

Scanning electron micrographs of *S. mutans* and *C. albicans* adhesion to the polystyrene substrata after 2h are shown in **Figs. 8** and **9**, respectively.

Fig. 8
S. mutans panel depicting adhesion phases. The panel displays SEM micrographs of *S. mutans* cells adherent to the polystyrene surfaces after 2 h, in presence of the following TSB-diluted solutions: A: D-glucose; B: L-glucose, C: xylitol, D: sorbitol. SEM micrographs showed scantily adherent yet uniformly distributed bacteria on polystyrene surfaces in all solutions, with relatively more organisms attached with the D-glucose solution than with the other solutions.

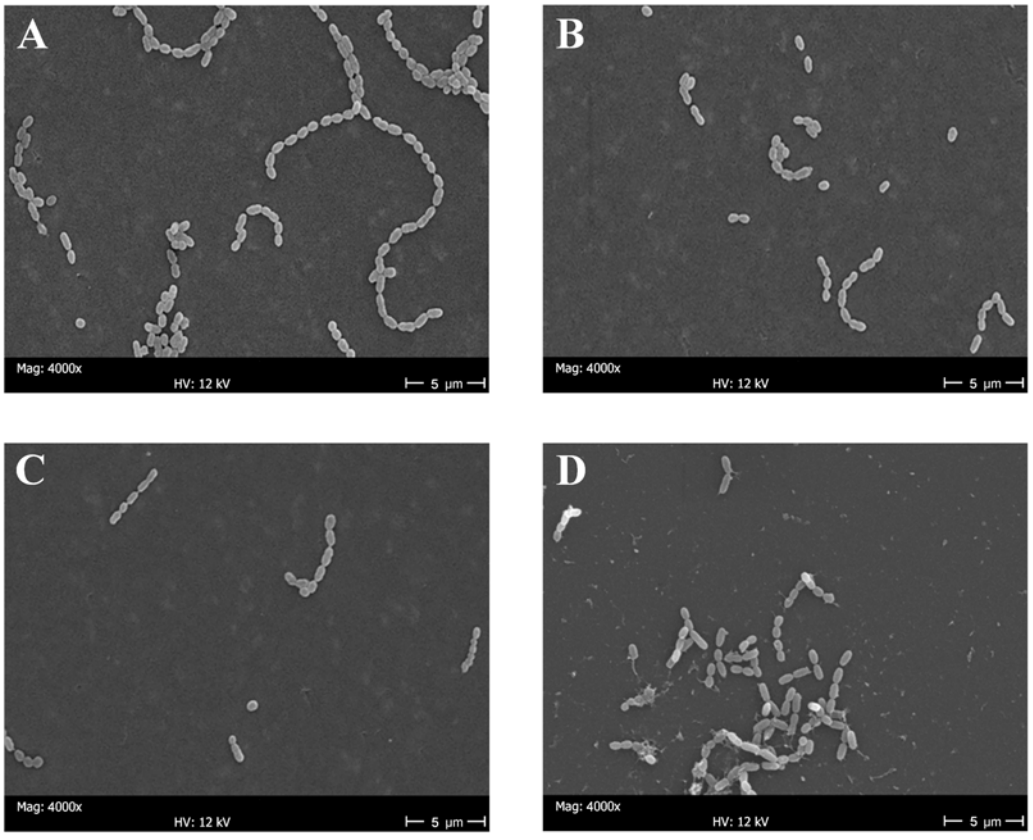
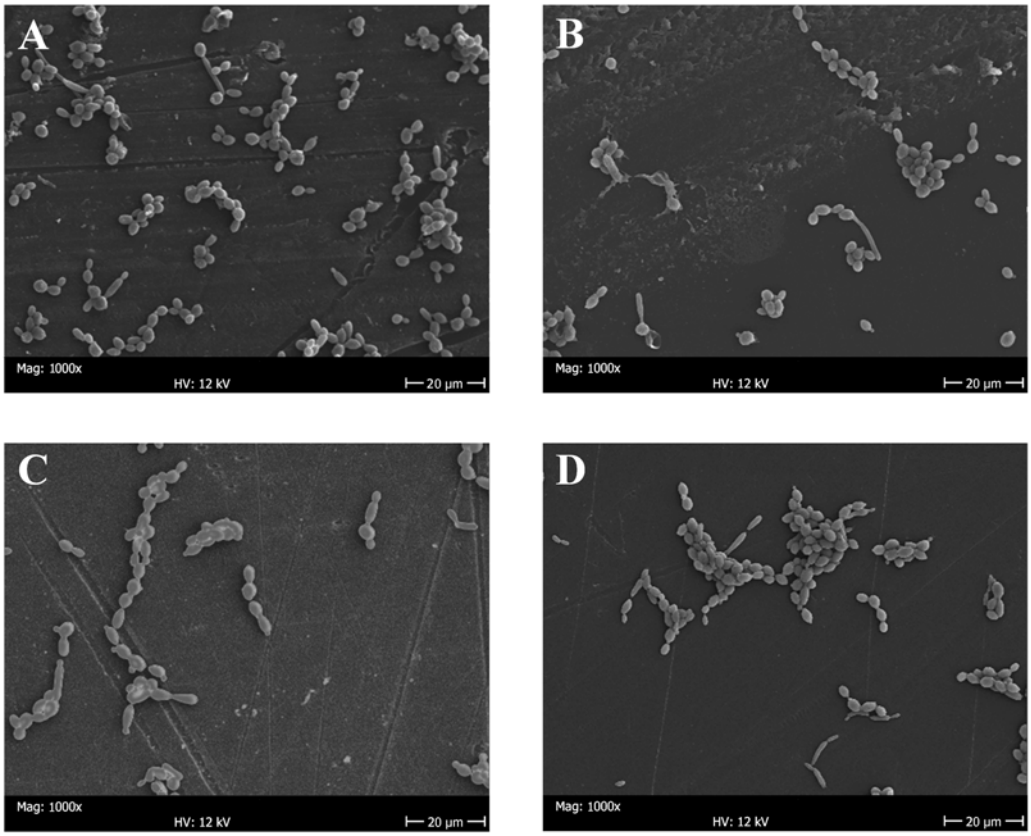


Fig. 9
C. albicans panel depicting adhesion phases. The panel displays SEM micrographs of *C. albicans* cells adherent to the polystyrene surfaces after 2 h, in presence of the following TSB-diluted solutions: A: D-glucose; B: L-glucose, C: xylitol, D: sorbitol. D-glucose elicited a greater degree of adhesion and a number of actively replicating and budding blastospores when compared to the other solutions. In all solutions and in particular in L-glucose and xylitol a low number of filaments composed of elongated cells that are attached end-to-end could be spotted, referring to initial hyphae formation.



SEM micrographs confirmed the biochemical data and showed scantily adherent yet uniformly distributed bacteria on polystyrene surfaces in all solutions, with relatively more organisms attached in D-form solutions than in L-form or polyol solutions. On microscopy *C. albicans* adhesion trends

reflected the foregoing quantitative data. Thus, D-carbohydrates appeared to elicit a greater degree of adhesion than the xylitol and L-carbohydrates solutions. A number of actively replicating and budding blastospores were seen in the D-solutions compared with the L-solutions and xylitol. In all solutions, and in particular in L-solutions and xylitol, a low number of filaments composed of elongated cells that are attached end-to-end could be spotted, referring to initial hyphae formation.

SEM evaluation of *S. mutans* and *C. albicans* biofilms after 24 h growth in the TSB-diluted tested carbohydrate solutions are shown in **Figs. 10** and **11**, respectively.

Fig. 10

S. mutans panel depicting biofilm formation phases. The panel displays SEM micrographs of *S. mutans* biofilm formation after 24 h, in presence of the following TSB-diluted solutions: A: D-glucose; B: L-glucose, C: xylitol, D: sorbitol. A multilayer, structured biofilm of *S. mutans* comprising numerous bacterial chains can be observed in all tested solutions.

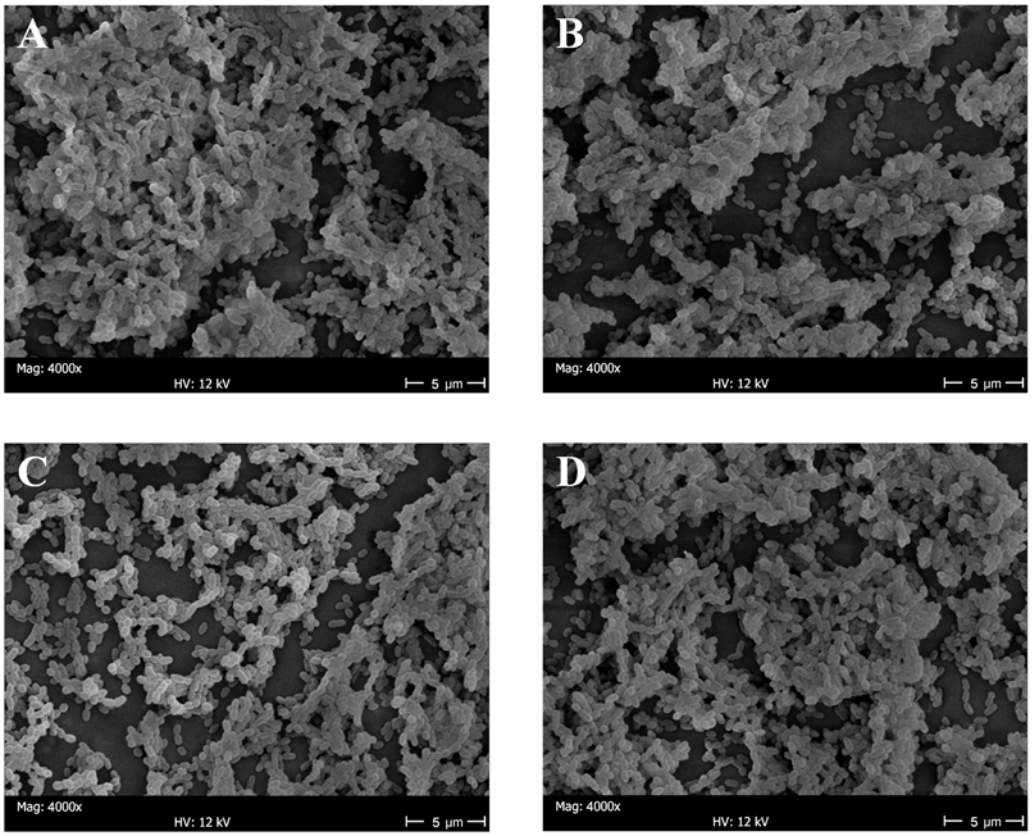
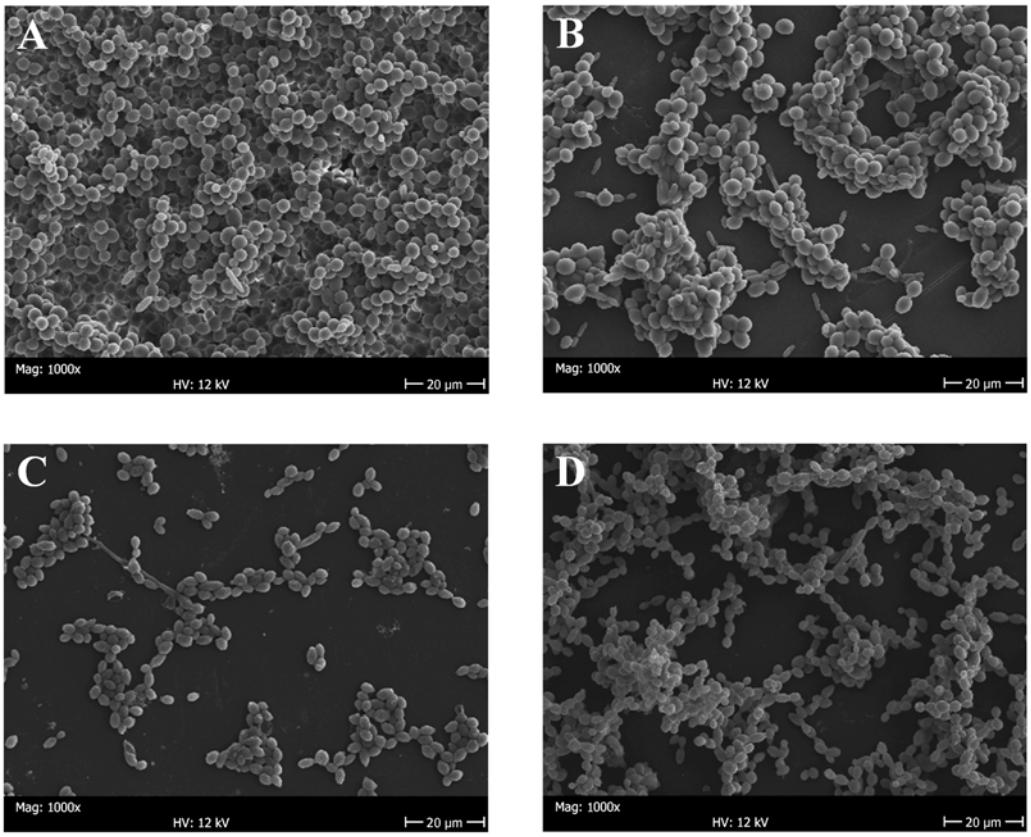


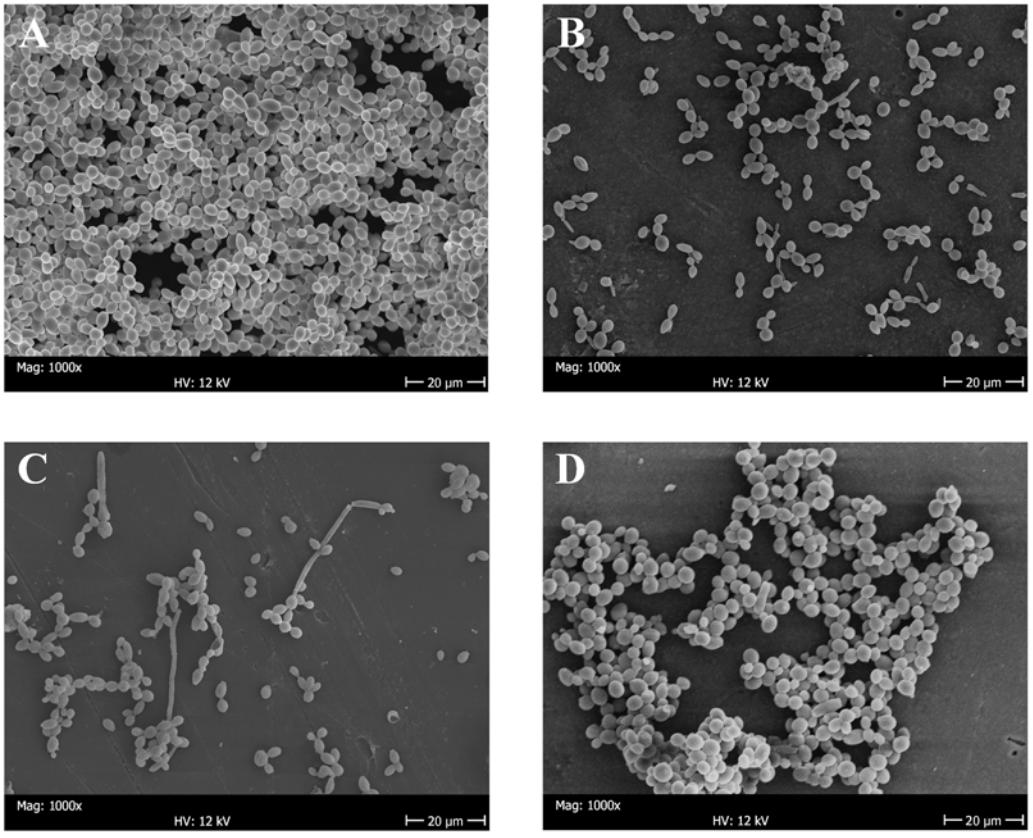
Fig. 11
C. albicans panel depicting biofilm formation phases. The panel displays SEM micrographs of *C. albicans* biofilm formation after 24 h, in presence of the following TSB-diluted solutions: A: D-glucose; B: L-glucose, C: xylitol, D: sorbitol. C. albicans biofilm specimens in the D- glucose solution showed a complete and multilayered surface coverage by blastospores showing budding processes. For the other tested solutions, the surface growth showed congregated groups of yeast cells and it was scanty when confronted to the D-glucose solution.



A multilayer, structured biofilm of *S. mutans* comprising numerous bacterial chains was observed after 24 h in all tested solutions. *C. albicans* biofilm specimens in the D-carbohydrates solutions (**Fig. 11a**), and in the raceme solutions showed a complete and multi-layered surface coverage by blastospores showing budding processes. For all the other tested solutions, the

surface growth showed congregated groups of yeast cells and it was scanty when confronted to the D-carbohydrate-containing solutions. *C. albicans* biofilms after 24 h growth in glucose-depleted Lee's medium are shown in **Fig. 12**.

Fig. 12
C. albicans panel depicting biofilm formation phases. The panel displays SEM micrographs of *C. albicans* biofilm formation after 24 h, in presence of the following glucose-depleted Lee's medium-diluted solutions: A: D-glucose; B: L-glucose, C: xylitol, D: sorbitol. D-glucose elicited a profuse biofilm development mainly consisting of blastospores showing budding processes. On the contrary, L-glucose and xylitol showed low-biofilm formation with more frequent signs of hyphal development compared to the same solutions prepared using TSB. Sorbitol showed similar biofilm formation to L-glucose and xylitol, but with less signs of hyphal development



Both tested D-carbohydrates and their corresponding raceme solutions elicited a profuse biofilm development mainly consisting of blastospores showing budding processes.

On the contrary, the control solution (glucose-depleted Lee's medium), the L-forms and xylitol showed low biofilm formation, with more frequent signs of hyphal development compared to the same solutions prepared using TSB (**Fig. 11b and c**). This is in keeping with the starvation induced by glucose depletion in Lee's medium which is one of the conditions promoting hyphal development. Sorbitol (**Fig. 11d**) and mannitol showed similar biofilm formation to L-sugars and xylitol but with less signs of hyphal development.

2.2.6 Discussion and conclusions

In this study, we evaluated the *in vitro* effect of a group of L- and D-carbohydrates, as well as three different polyols on *S. mutans* and *C. albicans* adhesion and biofilm formation.

A preliminary test was performed for both tested strains in order to find out which was the effect of changing the sugar concentrations to achieve the optimum growth conditions. *C. albicans* biofilms are usually obtained using a suitable medium additioned with either 100, 250, or 500 mM of D-Glucose depending on the different studies.(Jin, Samaranayake, Samaranayake, & Yip, 2004; Nikawa, Nishimura, Hamada, Kumagai, & Samaranayake, 1997; Samaranayake & MacFarlane, 1982) This corresponds to 1.8, 4.5% wt., and

9% wt. D-glucose concentration, respectively. *S. mutans* biofilm formation is mainly obtained in 1% wt. sucrose- or glucose-enriched medium. This sugar concentration elicits the maximum production of insoluble extracellular glucans, which is a sign of strong biofilm formation and increased pathogenicity.(Klein et al., 2010)

Therefore, we performed preliminary testing of biofilm formation for both strains with several concentrations (10, 5, 2.5, and 1.25% wt.), chosen in a geometric sequence with common ratio 2. A 5% wt. D-glucose addition resulted in good biofilm formation for both *C. albicans* and *S. mutans* using TSB as culture medium. Interestingly, Klein et al.(Klein et al., 2010) observed that *S. mutans* biomass decreased when a concentration of sucrose higher than 2% was used. The different response to increasing sugar concentrations may be explained by differences in the tested sugar (sucrose vs. glucose), substrata (saliva-coated hydroxyapatite vs. polystyrene), culture media (UF T YE vs. TSB) or, more likely, in *S. mutans* strains (UA159 vs. ATCC 35668). As a consequence of this preliminary test, only data regarding 5% wt. addition for every tested substance are shown in the following experiment, in order to be able to make immediate comparisons between the substances.

Results indicate that all levorotatory sugars, (either alone or in racemic combinations) were able to reduce *S. mutans* and *C. albicans* adhesion and biofilm formation *in vitro*, compared with their dextrorotatory counterparts. Furthermore, the polyols derivatives of the tested carbohydrates significantly

reduced *C. albicans* biofilm formation as well.

Both of the evaluated species showed a similar response on exposure to the tested solutions when cell adhesion was investigated: i.e., high-adhesion values with dextrorotatory carbohydrates and their corresponding polyols, intermediate values with the raceme solutions and low values with the levorotatory forms and xylitol. These findings confirm the observations of Samaranayake et al. (Samaranayake, McCourtie, & MacFarlane, 1980) who in their seminal studies for the first time demonstrated the enhanced adhesion of *C. albicans* grown in D-glucose. Nevertheless, in our study, xylitol was found to elicit the lowest values for *C. albicans* adhesion, unlike the findings of the aforementioned study. It must be noted, however, that the experimental design of this study differed from that of Samaranayake et al. (Samaranayake et al., 1980) as we directly performed the experiments in the presence of the tested sugars without pre-incubation of the organisms as in the previous investigation.(Samaranayake et al., 1980)

An enhancement of *S. mutans* adhesion by D-glucose was not unexpected, as this sugar induces extracellular glucan synthesis.(E. Soderling, Alaraisanen, Scheinin, & Makinen, 1987) Indeed, glucans are a key ingredient of the extracellular polysaccharide matrix of the biofilm. In one of the earliest studies, Soderling et al. (E. Soderling et al., 1987) demonstrated that sorbitol-enhanced *S. mutans* adhesion when compared with xylitol, confirming our findings.

The intermediate values of cell adhesion obtained for both strains with the

raceme solutions is interesting. This may be explicable in terms of a reversible process of competitive inhibition of the adhesins on the cell surfaces, wherein L-carbohydrates bind to cell adhesins. However, further studies are needed to confirm this hypothesis.

It should be noted that we grew both species initially in BHI which contained glucose. Therefore, when the organisms were transferred to new carbon sources, a lag period could be expected until the microbes acclimatized to the new environment and altered their gene expression. This phenomenon may have had a significant impact on adhesion and might explain why, for instance in the *S. mutans* strain, sorbitol, and D-mannose adhesion values were significantly lower than that of D-glucose. The latter hypothesis, however, does not hold true for the tested yeast strain, since adhesion values of Candida for sorbitol and D-mannose were not significantly different from that of D-glucose. The two tested species showed dissimilar behavioral patterns for biofilm formation probably due to their differential metabolic activity. It is well known that the prokaryote *S. mutans* is remarkably versatile in the range of carbohydrates which it can utilize and this feature enables it to outgrow other species when the diet is rich in carbohydrate, regardless of the particular sugars present. (Colby & Russell, 1997; Forssten, Bjorklund, & Ouwehand, 2010)

Our data supports the previous findings of Colby and Russell(Colby & Russell, 1997), since further carbohydrates or polyol supplements (to a concentration of up to forty times that of TSB glucose content), increased streptococcal biofilm

growth to only 30% greater than the values of the controls. Our data are also in keeping with the findings of Brown et al., who demonstrated that *S. mutans* is able to ferment both sorbitol and mannitol by a pathway that involves distinct, inducible enzymes.(Brown & Wittenberger, 1973) On the contrary, in the eukaryote, *C. albicans* biofilm growth was remarkably enhanced by D-glucose and D-mannose, these carbohydrates were able to promote *C. albicans* biofilm formation to about 500% the values of TSB. These observations may be explained by an up-regulation of carbon metabolism of the yeast, as described by Han et al. (Han, Cannon, & Villas-Boas, 2011) In fact, the latter authors noted that central carbon metabolism apart from being responsible for supplying carbon and lipid sources for cellular building blocks, is also responsible for the biosynthesis of quorum sensing molecules involved in cell–cell communication within the biofilm.

As mentioned above, akin to adhesion experiments, a reversible mechanism of competitive inhibition of specific enzymes in the metabolic pathway of the dextrorotatory carbohydrates may explain the intermediate values for biofilm formation we noted in both species with the raceme solutions.(Moazeni et al., 2010) (Sun, Saccomanno, Hedlund, & McKay, 2009) Future studies are, nevertheless, needed to explore this hypothesis.

Despite the fact that *C. albicans* is known to be able to rapidly synthesize both sorbitol and mannitol as a stress response mechanism(Sanchez-Fresneda et al., 2013), it was surprising to view that the tested concentrations of both polyols

when diluted in TSB did not elicit a significant increase in biofilm formation. When diluted in glucose-depleted Lee's medium, sorbitol significantly increased the yeast biomass to 161% of the control, therefore, it may be reasonable to speculate that a medium which facilitates hyphae formation may also lead to an induction of polyol metabolic pathways in the tested yeast. It was indeed demonstrated that both a sugar and a nitrogen (amino acids) source are necessary for *C. albicans* morphogenesis.(Holmes & Shepherd, 1988) The much lower biomass observed when incubated with sorbitol than with D-glucose solubilized in Lee's medium may be explained by a lag period needed by the yeast cells to up-regulate the metabolic pathways necessary for sorbitol and, to a lesser extent, mannitol catabolism.

Levorotatory sugars led to the lowest levels of biofilm formation in both species, implying that the two tested microorganisms had difficulty in metabolizing these nutrients. In a previous study, Moazeni et al. (Moazeni et al., 2010) described the dissimilar ability of different microorganisms to selectively metabolize carbohydrates. It is intriguing in evolutionary terms, why some microorganisms, more than others are naturally selected to metabolize only certain enantiomeric forms of carbohydrates.(Moazeni et al., 2010)

Our results further imply that xylitol induces lowest adhesion in both species, although it elicited similar degree of biofilm formation with L-carbohydrates. Xylitol is a well-known polyol whose caries-suppressive activity has been

extensively documented. (Milgrom, Soderling, Nelson, Chi, & Nakai, 2012; E. M. Soderling, 2009) Up to now only a few workers have evaluated the effect of xylitol on *C. albicans* adhesion and biofilm formation.(Ichikawa et al., 2008; Samaranayake & MacFarlane, 1982; Samaranayake et al., 1980) Although xylitol suppressed the adhesion of both the tested species in the current study, this effect was not evident during prolonged growth and biofilm formation. A number of others have previously confirmed these findings.(Fontana & Gonzalez-Cabezas, 2012; Ichikawa et al., 2008)

In translational terms, as commercial mass-production of xylitol is much more cost-effective than glucose and sucrose, further research is warranted to study whether xylitol could be used as a plaque biofilm suppressant in combination with other plaque reducing agents, such as triclosan, for commercial purposes. The surface properties of substrata are known to significantly influence biofilm development of both the tested species.(Ionescu et al., 2012; Lamfon, Porter, McCullough, & Pratten, 2003) The substratum used in this study for adhesion and biofilm formation was polystyrene. It is a synthetic polymer and was chosen due to its high surface uniformity and its propensity to nurture satisfactory bacterial and yeast colonization.(Li et al., 2010; Monteiro et al., 2011) In addition, Li et al. (Li et al., 2010) also reported similar levels of *S. mutans* and *C. albicans in vitro* biofilm development on polystyrene surfaces as wells as on polymethylacrylate denture resin surfaces. It is tempting to speculate, therefore, that levorotatory sugars may suppress candidal

colonization in denture wearers, leading to a resultant reduction of Candida-associated denture stomatitis – a chronic problem in elderly denture wearers.

All oral surfaces are intrinsically coated by a thin salivary pellicle. Previous studies have nevertheless reported conflicting results on the influence of a salivary pellicle on the adhesion and biofilm formation of both *C. albicans*(Jin et al., 2004) (McCourtie, MacFarlane, & Samaranayake, 1986; Nikawa et al., 1997) and *S. mutans*.(Ahn, Lim, & Lee, 2010; Pratt-Terpstra, Weerkamp, & Busscher, 1987)

Although some investigators suggest that the surface properties of the original substrata are transferred even through a salivary protein layer(Pratt-Terpstra et al., 1987), it can only be estimated how the presence of a salivary pellicle would have influenced biofilm formation in the present study. Salivary pellicle is thought to play a significant role more during adhesion and early colonization phases than during biofilm formation(Busscher, Rinastiti, Siswomihardjo, & van der Mei, 2010), yet a study demonstrated that surface free energy influenced *S. mutans* adhesion irrespective of saliva coating.(Ahn et al., 2010) The use of a single substratum with high-surface uniformity for all cultures and the aforementioned findings can explain why the effect of a saliva coat was not evaluated in the current system, however, it may be interesting for further studies to address this issue, since, especially during adhesion phases, L- and D-rotatory carbohydrates might interact in different, specific ways with the salivary pellicle rather than with the polystyrene substratum.

The results of the present study should also be interpreted within the study's limitations, including its *in vitro* character and the simulation of single-species biofilm formation. It is well known, in fact, that multi-species biofilms do rather respond to the oral cavity. It is also ascertained that a high-dietary intake of D-rotatory carbohydrates can drive a shift in the composition and metabolic activity of the biofilm leading to disbyosis and the onset of pathological conditions. Interactions between species inside a biofilm community, known as quorum sensing, are complex and also involve carbohydrates as signaling molecules. Finally, as previously specified exogenous carbohydrates are able to prevent microbial adhesion to epithelial cells by competitively inhibiting microbial lectins. All these circumstances suggest that trying to explain at a molecular level the effect that the sugars tested in the present study may have on a multi-species biofilm, be it *in vitro* or *in vivo*, may be very challenging. Nevertheless, trying to understand these effects may be of primary importance from a point of view of a dietary control of dysbiotic oral biofilms.

In conclusion, our results elucidate a hitherto poorly described metabolic interactions of two individually tested oral commensals, *S. mutans* and *C. albicans*, with a group of L- and D-rotatory carbohydrates and polyols, and how these interactions impact on their oral colonization specifically in terms of adhesion and biofilm formation. In translational terms, the foregoing data may have practical implications, and raises the interesting possibility of dietary control and prevention of dysbiotic oral biofilms using xylitol or levorotatory

carbohydrates.

2.3 Innovation in dental materials materials

2.4 Introduction

2.4.1 The issue of polymerization shrinkage and the introduction of siloranes in restorative dentistry

Polymerization shrinkage, which is caused by the conversion of monomer molecules into a polymer network(Peutzfeldt, 1997), is one of the main issues with RBCs. In fact, this process induces stresses both into the resin restorations and the surrounding tooth structure leading to microfractures and/or blistering, eventually leading to loss of marginal seal.(Braga & Ferracane, 2004; Ferracane & Mitchem, 2003; Kleverlaan & Feilzer, 2005; Suliman, Boyer, & Lakes, 1994; Yamazaki, Bedran-Russo, Pereira, & Swift, 2006) Two strategies have been used in order to overcome this drawback: lowering the number of reactive site per volume and using new resins.(Weinmann, Thalacker, & Guggenberger, 2005) Increasing the molecular weight of the monomers and the filler load are two methods to reduce the number of reactive sites but an augmented molecular weight can compromise the handling characteristics of resin composites and increase polymerization stress, while an overload of inorganic filler saturates the resin capacity to incorporate its particles.(Weinmann et al., 2005)

Since the introduction of the methacrylate-based chemistry in dentistry by

Bowen in 1965 different alternatives have been studied over time, some of them by Bowen himself.(Bowen, 1956) Research on epoxy resins has led to the development of a new kind of monomers, the siloranes.(Buergers, Schneider-Brachert, Hahnel, Rosentritt, & Handel, 2009; Eick et al., 2007; Ilie & Hickel, 2006) Silorane monomer represents a hybrid molecule, made of a central siloxane ring to which oxirane structural moieties are attached. The silorane matrix is formed by silorane monomers through a cationic ring-opening polymerization process. The opening of the epoxide rings compensates the polymerization shrinkage,(Ilie & Hickel, 2006) thus generating a material that possibly overcomes one of the main issues of modern RBCs.

Compared to methacrylate-based restorative materials, silorane-based composites show very low polymerization shrinkage but overall mixed mechanical performances. The silorane-based material has relatively higher flexural strength/modulus, fracture toughness, but rather lower compressive strength and microhardness than the methacrylate-based composites. (Duarte, Botta, Phark, & Sadan, 2009; Lien & Vandewalle, 2010; Weinmann et al., 2005)

2.4.2 The importance of light-curing resin-based composites

Restorative dentistry has shown an increasing use of resin-based composites (RBCs), thanks to their characteristics(Hickel, Dasch, Janda, Tyas, & Anusavice, 1998) until they have become the most used restorative material.(Demarco, Correa, Cenci, Moraes, & Opdam, 2012) RBCs are

generally cured by light-induced polymerization of monomers. Nevertheless, since complete polymerization of these materials never occurs,(F. Rueggeberg, 2005; F. A. Rueggeberg & Caughman, 1993; F. A. Rueggeberg, Hashinger, & Fairhurst, 1990; F. A. Rueggeberg & Margeson, 1990) monomers may leach out of composites.(Hagio, Kawaguchi, Motokawa, & Miyazaki, 2006; Nalcaci, Ulusoy, & Atakol, 2006; Polydorou, Trittler, Hellwig, & Kummerer, 2007; Takahashi, Imazato, Russell, Noiri, & Ebisu, 2004) Previous data showed that light-curing time of a RBC is a crucial factor in determining the characteristics of surface colonization.(Brambilla, Gagliani, Ionescu, Fadini, & Garcia-Godoy, 2009) This is a very important aspect since it leads to biofilm development, which is one of the most important factors in caries formation.(Marsh, 2006; Pereira-Cenci, Cenci, Fedorowicz, & Azevedo, 2013; Selwitz, Ismail, & Pitts, 2007) In fact, an imbalance of the oral microbial communities with an increase of cariogenic bacteria is considered the first step in both primary and secondary caries development.(Caufield, Li, & Dasanayake, 2005; Levato, 2005; Michalek, Katz, Childers, Martin, & Balkovetz, 2002; Tanzer, Livingston, & Thompson, 2001) *Streptococcus mutans* (*S. mutans*) represents the main microorganism responsible for caries lesions, hence influencing the restoration success over time.(Busscher et al., 2010; Kuramitsu & Wang, 2011; Lobo, Goncalves, Ambrosano, & Pimenta, 2005; Selwitz et al., 2007)

2.4.3 Influence of light-curing parameters on biofilm development and flexural strength of a silorane-based composite

No previous studies have investigated biofilm development and flexural strength of silorane-based composites as a function of their curing parameters. Therefore, it was interesting to evaluate these aspects which can play a pivotal role in increasing the survival of composite restorations thus reducing the need of more invasive treaments in the long term.

2.4.4 Aim of the study

The aim of this study was to assess the influence of curing time and power on the *in vitro S. mutans* biofilm development and on the flexural strength of a silorane-based composite. The tested null hypothesis was that the silorane-based composite would not present different biofilm development and flexural strength from the methacrylate-based composite.

2.4.5 Materials and Methods

All reagents and multi-well plates used in the present study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Two commercially available RBCs, based on either silorane (Filtek Silorane; 3M ESPE, Seefeld, Germany) or methacrylate-based resin chemistry (Filtek Z250; 3M ESPE, Seefeld, Germany) were used in this study. The shade used was A3, composition is found in **Table 3**.

Table 3

Composite resins composition according to manufacturer

Composite	Organic matrix	Filler
Filtek Silorane	Silorane (3,4-epoxycyclohexylethylcyclopolymethylsiloxane,bis-3,4-epoxycyclohexylethylphenylmethylsilane)	Silanized quartz, yttrium fluoride
Z250	Bis-GMA, UDMA, Bis-EMA	Zirconia/silica

Specimen preparation for the microbiological procedures

The wells of a 96-well polystyrene plate were separated from the base of the plate and used as moulds to create standardized test disks (6.4mm diameter and 1.5 mm thickness). For the preparation of a single RBC test specimen, an excess amount of uncured resin-based composite was placed in a single trimmed well, covered with a Mylar strip to prevent the formation of an oxygen-inhibited layer, and then condensed against a glass plate. The disks were randomly divided into six groups and light-cured for 10, 20, 30, 40, 60 and 80 s, respectively using a light-curing unit (Spectrum 800, DENTSPLY International Inc., York, PA, USA). The light-curing unit was set at two light-curing intensities (400 and 800 mW/cm^2), thus generating two sub-groups differing in the light-curing intensity for each time group. The light-guide end was placed directly in contact with one of the two mylar strips covering the composite surface. A total of 18 disks for each curing time group and light-curing

intensity subgroups were produced. After the polymerization the specimens were carefully removed from the wells and checked for visible surface irregularities. No finishing procedure was adopted. The plates were stored in a dark place for 24 hours at 37°C to allow complete polymerization of the disks. Two-hundred microliters of sterile phosphate buffered saline (PBS) were then placed in each well and the plates were stored for additional 7 days to allow the leaching of most of the residual monomers. In order to remove the leached monomers, each well was washed twice every day using sterile PBS. Subsequently, 16 disks for each group were transferred in new 96-well polystyrene plates. These plates were then sterilized using a chemiclave with hydrogen peroxide gas plasma technology (Sterrad; ASP, Irvine, CA, USA). By limiting the maximum temperature to 45°C, heat-related damage of the RBC specimens was avoided.

Bacteria

All the culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA). A pure suspension of *Streptococcus mutans* strain ATCC 35668 in brain-heart infusion broth (BHI) was obtained after a 12 h incubation at 37°C in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (2.200 rpm, 19 °C, 5 min), washed twice with sterile PBS and resuspended in the same buffer. The cell suspension was subsequently subjected to low intensity ultrasonic energy in order to disperse

bacterial chains, and the optical density was adjusted to 0.3 OD units at 550 nm (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA), which corresponds to a microbial concentration of 3.65 x 10^8 cells/mL.

MTT assay reagents

A tetrazolium salt (MTT) stock solution was prepared by dissolving 5 mg/ml of [3 - (4, 5) dimethylthiazol -2-yl) - 2, 5 diphenyl tetrazolium bromide] in sterile PBS; a phenazonium salt (PMS) stock solution was prepared by dissolving 0.3 mg/ml of N-methylphenazonium methyl sulphate in sterile PBS. The solutions were stored at 2°C in light-proof vials until the day of the experiment, when a fresh measurement solution (FMS) was made by mixing 1 mL of MTT stock solution, 1 mL of PMS stock solution and 8 mL of sterile PBS.

A lysing solution (LS) was prepared by dissolving 10% V/V of sodium dodecyl sulphate (SDS) and 50% V/V of dimethylformamide (DMF) in distilled water.

Saliva collection

Unstimulated saliva from three healthy donors was used in this study according to Guggenheim and others.(Guggenheim, Giertsen, Schupbach, & Shapiro, 2001) Saliva was collected in chilled test-tubes, pooled, heated at 60°C for 30 min to inactivate endogenous enzymes and then centrifuged (12.000 x g) for 15

min at 4°C. The supernatant was transferred in sterile 10 ml tubes, then stored at -20 °C. Immediately before starting the experimental session, saliva was thawed at 37 °C for 1h. One hundred microliters of saliva were placed into each well of the specimen-containing sterilized plates; the plates were incubated for 4 hours at 37°C. Then, the saliva was blotted out and the wells were gently rinsed twice with 200 µl of sterile PBS.

Biofilm development

Twenty microliters of the bacterial suspension in early log phase and 180 µl of sterile BHI were placed in each well. The plates were incubated 24 h at 37° C in a 5% supplemented CO_2 environment to allow biofilm development. The culture was then discarded and the wells were carefully washed twice with sterile PBS in order to remove non-adherent cells.

MTT assay

Specimen-containing plates were filled with 100 µl of MTT solution for each well; the plates were incubated for 3 h in a dark place at 37°C: during incubation, microbial redox systems converted the yellow salt to intracellular insoluble purple formazan. Then MTT solution was gently discarded and the intracellular formazan crystals were dissolved by adding 100 µl of lysing solution to each well and incubating again for 1 h at room temperature in a dark place. Finally, 90 µl of suspension were taken from each well and its

absorbance was measured with a spectrophotometer at 550 nm (Genesys 10-S) and expressed as optical density (OD) units.

Laser confocal microscopy (CLSM)

Two disks for each experimental group were prepared for CLSM analysis. However, due to the number of specimens groups, it was decided to analyze only the 10 and 80 s curing time groups at 400 mW/cm^2 curing intensity. This decision was taken after the MTT results were obtained as they highlighted a major difference in biofilm development between these curing time groups. After the 24 h incubation, the biofilm growing on the disks was gently washed with PBS to remove non adherent cells and stained using the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit for microscopy (Invitrogen Ltd., Paisley, UK). The fluorescence from stained cells adherent to the samples was observed using a CLSM (Leica TCS SP2, Leica microsystems, Wetzlar, Germany). Four randomly selected image stack sections were recorded for each biofilm specimen. Confocal images were obtained using a dry 20x (NA = 0.7) objective and digitalized using the Leica Application Suite Advanced Fluorescence Software (LAS AF, Leica microsystems, Wetzlar, Germany) at a resolution of 1024x1024 pixels, with a zoom factor of 1.0. For each image stack section an average intensity projection (AIP) and a 3D reconstruction were obtained. AIPs were done using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) while Drishti (http://sf.anu.edu.au/Vizlab/drishti/) was used

for 3D reconstructions.

Specimen preparation and flexural strength evaluation

A modified procedure from the ISO 4049/2000 guidelines was used for the flexural strength evaluation. Briefly, a 2 mm thick polyether strip (Impregum, 3M ESPE AG, Seefeld, Germany) was obtained; the strip was then cut in order to obtain multiple bar-shaped standardized holes with a length of 10 mm and a width of 2 mm. For the preparation of RBC test specimens an excess amount of uncured resin-based composite was placed in the standardized holes, covered with Mylar strips to prevent the formation of an oxygen-inhibited layer and then condensed against a glass plate in order to remove excess material. The glass plate was then removed, the bars from each tested RBC were randomly divided into six groups and light-cured for 10, 20, 30, 40, 60 and 80 s, respectively using a light-curing unit (Spectrum 800). The light-curing unit was set at two light-curing intensities (400 or 800 mW/cm^2), thus generating two sub-groups differing in the light-curing intensities for each time group. The light-guide end was placed directly in contact with one of the two mylar strips covering the RBC surface. A total of 8 bars for each light-curing time and light-curing intensity subgroup were produced. After polymerization the specimens were carefully removed from the strip, checked for visible surface irregularities and stored in a dark place for 24 hours at 37°C to allow complete polymerization.

After that, the bars were submitted to a three-point bend test with a universal testing machine with a crosshead speed of 1 mm/min. The maximum loads at fracture were obtained and the flexural strength (σ) was calculated in megaPascals (MPa) by using the following formula: $\sigma = 3FL/(2BH^2)$ where F is the maximum load (in Newtons); L is the distance between the supports (in millimeters); B is the width of the specimen (in millimeters) and H, the height (also in millimeters). The formula was solved assuming that the prepared bars had a 2 mm width and a 2 mm thickness, and the custom-made support for the bars had a distance L equal to 8 mm.

Statistical analysis

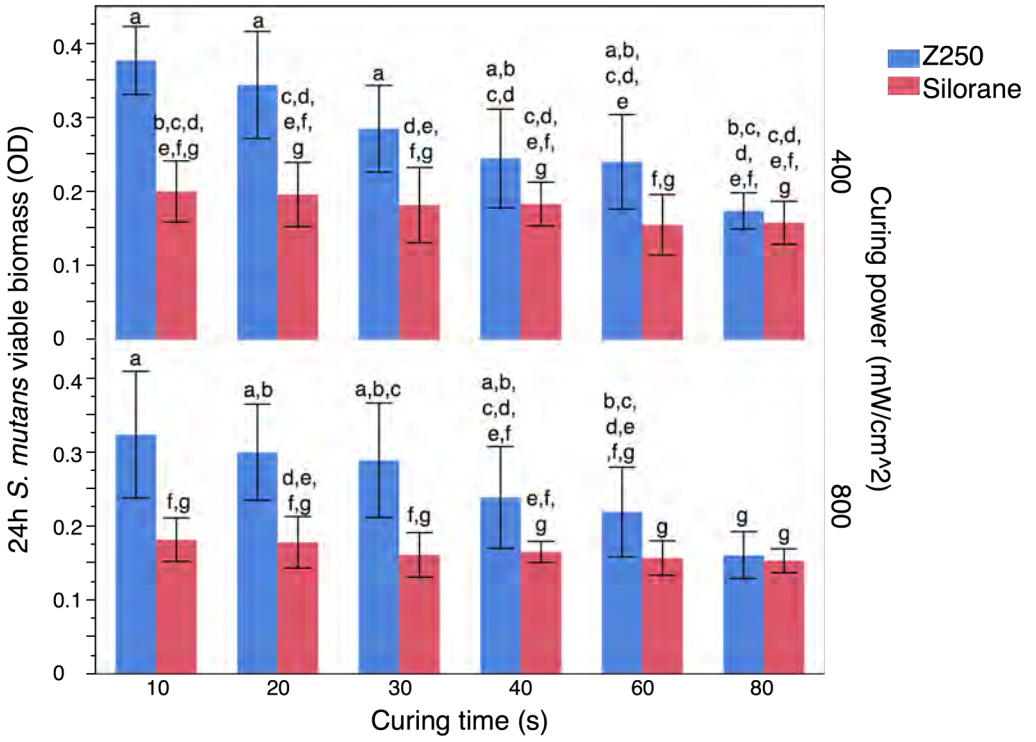
All statistical analyses were performed using statistical software (JMP 10.0, SAS Institute, Inc., Cary, NC, USA). OD and flexural data are reported throughout the text as means and standard deviations (SD) calculated from the natural values.

Data were analyzed by three-way ANOVA with balanced data in which light-curing time (6 levels, i.e., 10, 20, 30, 40, 60, or 80 s), light-curing intensity (2 levels, i.e., 400 or 800 mW/cm^2) and resin composite type (2 levels, i.e., methacrylate or silorane-based composites) were fixed factors. Homogeneity of variances was preliminarily checked using Bartlett's test. Tukey's post-hoc test was used to highlight significant differences ($p<0.05$).

2.4.6 Results

Experiment 1: Biofilm development on resin composite discs expressed as mean OD ± 1SD is reported in **Fig. 13**.

Fig. 13
Biofilm development expressed as OD. Bars represent means and error bars represent SD. Different superscript letters stand for statistically significant differences



Methacrylate-based and silorane-based composites showed significantly different biofilm development ($p<0.0001$). Light-curing time ($p<0.0001$) and light-curing intensity ($p<0.0108$) were also found to be significant factors in influencing biofilm development.

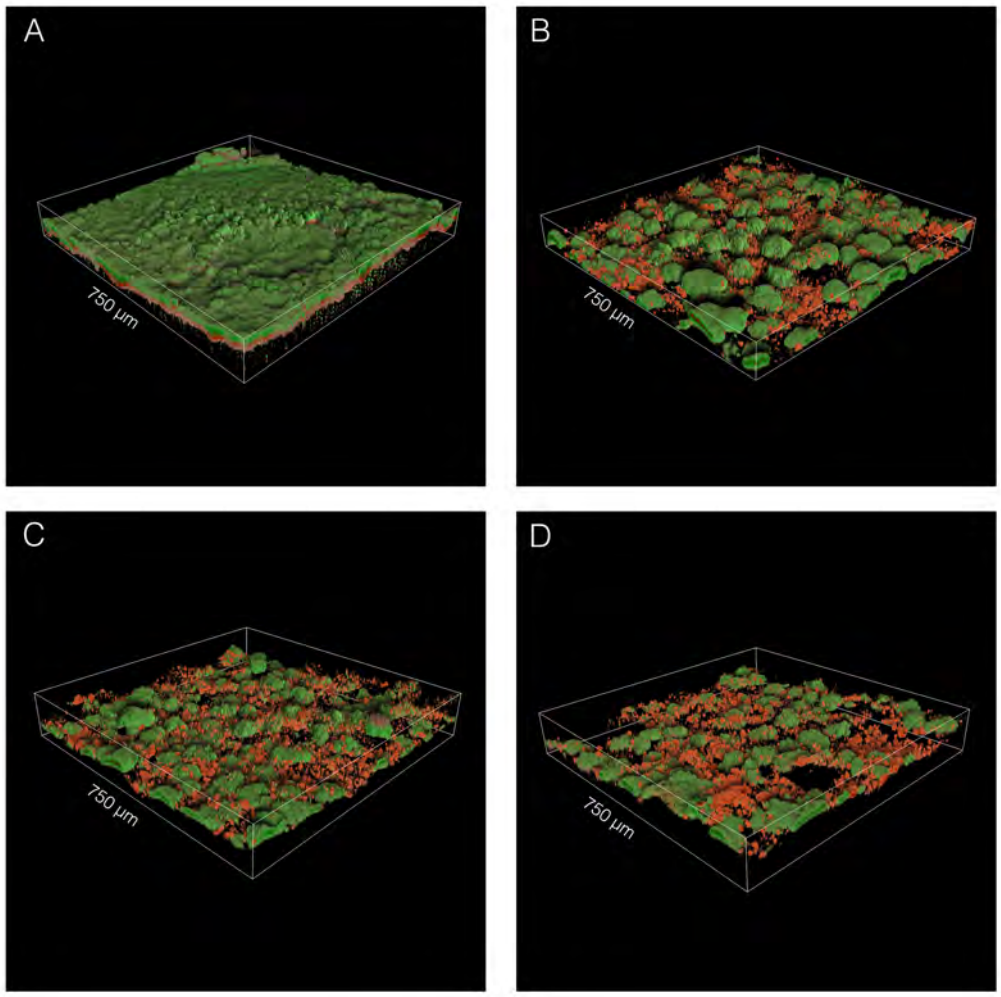
For each light-curing time and light-curing intensity group except 80 s,

silorane-based composite demonstrated lower biofilm development when compared to methacrylate-based one. Extended light-curing times and higher light-curing intensities showed a reduction in OD values for both resin composites. However, this phenomenon proved to be significant only for the methacrylate-based resin composite.

The lowest biofilm development was obtained on the surfaces of silorane-based composite light-cured for 80s at 800 mW/cm^2 light-curing intensity, while the highest biofilm development was obtained with methacrylate-based composite light-cured for 10s at 400 mW/cm^2 light-curing intensity.

As we can see from **Fig. 14**, the methacrylate-based composite light-cured for

Fig. 14
Confocal laser scanning microscopy 3D reconstructions of the biofilms, which were stained with live/dead stain. Green represents live bacteria and red represents non-viable, dead bacterial cells. Letters refer to the different experimental groups: A= 10 s 400 mW/cm^2 methacrylated-based composite, B= 80 s 400 mW/cm^2 methacrylated-based composite, C= 10 s 400 mW/cm^2 silorane-based composite, D= 80 s 400 mW/cm^2 silorane-based composite



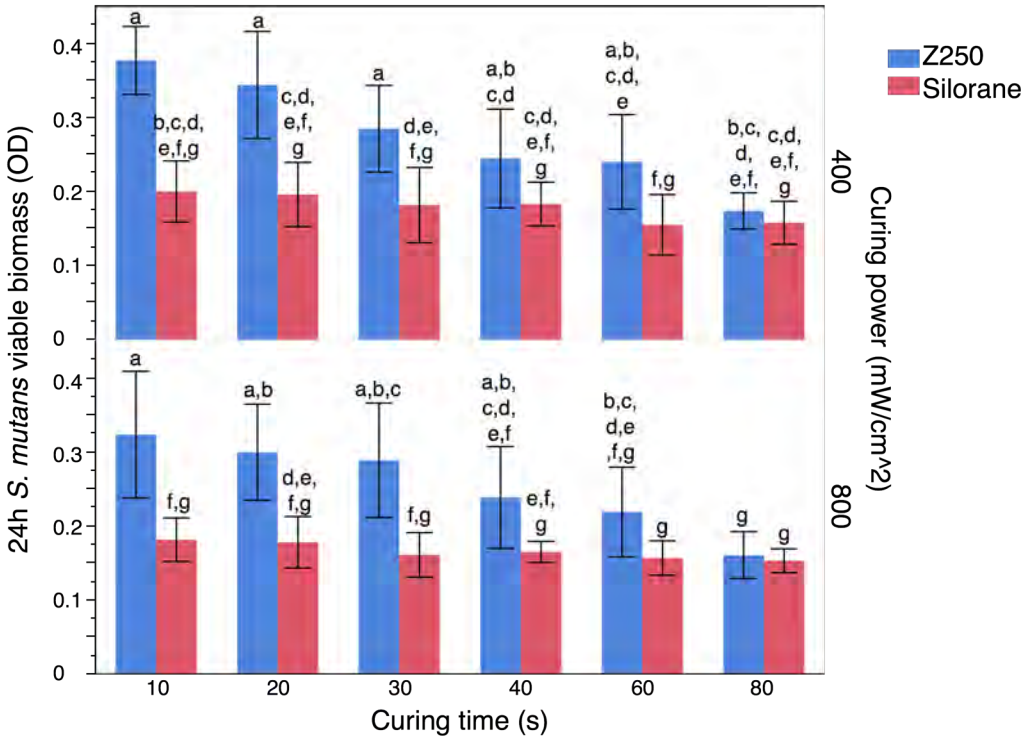
80s at 400mW/cm^2 light-curing intensity and silorane-based composite light-cured for 10 s and for 80 s at 400mW/cm^2 light-curing intensity resulted in similar biofilm development with several live (green) and dead (red) S. mutans colonies covering the surface of the samples.

Differently, the methacrylate-based composite light-cured for 10s at 400mW/cm^2 light-curing intensity showed an increased biofilm development with most of the surface covered by live (green) *S. mutans* colonies.

Flexural strength

Experiment 2: Flexural strength expressed as mean MPa ± 1SD are shown in **Fig. 15.**

Fig. 15
Flexural strength expressed as MPa. Bars represent means and error bars represent SD. Different superscript letters stand for statistically significant differences



Three-way ANOVA did not show any interaction among the considered factors, therefore analysis was performed for each factor according to one-way ANOVA model. Flexural strength was only influenced by composite type ($p<0.0318$), with methacrylate-based composite showing higher flexural strength than silorane-based composite. In particular, the best results were obtained by methacrylate-based composite at 80s light-curing time and 800 mW/cm^2 light-curing intensity; however, they were not significantly different from the other methacrylate-based composite sub-groups. Considering silorane-based composite the best results were obtained for the 80s 400 mW/cm^2 group, no significant differences between the other silorane-based composite sub-groups were observed.

2.4.7 Discussion

Silorane-based composites were introduced as alternatives to conventional methacrylate-based ones in order to reduce polymerization shrinkage.(Hagio et al., 2006; Nalcaci et al., 2006; Polydorou et al., 2007; Takahashi et al., 2004) As bacterial colonization is an important factor for restoration longevity, the evaluation of the biological properties of these alternative resin composites seems to be another important issue to be investigated.

The best way to obtain data on bacterial colonization of the composite surface is to use an *in vitro* experimental model.(Coenye & Nelis, 2010; Ledder &

McBain, 2012; Andrew J. McBain, 2009; Wong & Sissons, 2001) In this study a Drip-flow reactor was chosen in order to achieve similar growth conditions for all resin specimens and to keep all the experimental parameters under controlled conditions.(A. J. McBain, 2009) Besides the experimental setup of the reactor, different parameters related to the material characteristics need to be considered: in particular the surface roughness (SR) and the curing process parameters. The influence of SR on biofilm development was excluded by polymerizing the specimens against a Mylar strip. This technique allowed to obtain specimens with a mean surface roughness R_a=0.06 µm (data not shown), which is below a 0.2 µm threshold introduced by Bollen and others in the 1990s.(Bollen, Lambrechts, & Quirynen, 1997) The results of this study suggested that R_a values below the specified threshold do not have a significant influence on biofilm development. Regarding the curing process, it has been demonstrated that light-curing time and light-curing intensity deeply influence biofilm development on resin composite surfaces.(Brambilla et al., 2009) Consequently it was decided to light-cure the specimens at six different times using two light-curing intensities to obtain data on the influence of these variables. While not of clinical relevance 80 s light-curing time was used in order to have a group of specimens with the highest degree of conversion possible.

The results of the study allow the rejection of the first null hypothesis since the silorane-based composite surfaces showed a reduction in biofilm development

when compared to the methacrylate-based ones ($p<0.0001$). In particular, methacrylate-based resin composite showed a decreasing colonization trend as light-curing time increased, agreeing with the results of a previously study.(Brambilla et al., 2009) On the contrary, silorane-based composite did not present significantly different values among light-curing times tested. These results allowed to suppose that physico-chemical properties such as surface roughness and hydrophobicity could influence material biological behavior. Since in this study the influence of surface roughness variation could be excluded, it is possible to suggest that silorane increased hydrophobicity make this material less susceptible to biofilm development.

As for light-curing time, a significant statistical difference ($p<0.018$) in biofilm development was found between the two tested light-curing intensities but only in methacrylate-based composite. This suggests that for these materials a better biological performance can be achieved by using the highest light-curing intensity tested (800 mW/cm^2).

The results of this study allowed a better comprehension of silorane-based composites tendency to biofilm development since no other authors worked on this topic. However, a previous article by Buergers and others (Buergers et al., 2009) demonstrated that silorane-based composites susceptibility to bacterial adhesion *in vitro* is lower than four conventional methacrylate-based composites. The article suggested that silorane-based composite matrix and in particular its hydrophobicity may negatively influence bacterial

adhesion(Buergers et al., 2009), thus confirming the hypothesis of our work. However, saliva was not used, as it was stated that the protocol was kept as simple as possible and that saliva was not the only factor differentiating an *in vitro* study from an *in vivo* study. Yet, another *in vitro* work didn't show differences between silorane and methacrylate-based composites bacterial adhesion.(Poggio et al., 2009) Nevertheless, composites SR values were too inhomogeneous to easily compare the different groups.(Poggio et al., 2009) Up to now only two *in situ* studies evaluated the biological performances of silorane-based composite. In the first study Claro-Pereira and others(Claro-Pereira et al., 2011) showed similar adhesion values for both silorane and methacrylate-based composites. Nevertheless, the presence of several variables difficult to control and the limit number of subjects involved, represent the weak points of this work. Instead, another *in situ* study evaluated the demineralization of dentin next to multiple restorative materials.(van de Sande et al., 2014) Results highlighted a high dentin demineralization associated with silorane-based composite. However it is difficult to understand how restorative materials without the incorporation of any antibacterial principle can influence dentin demineralization in their proximity. Moreover, in this study, specimens were kept in an acrylic resin full prosthesis whose oral flora is probably very different from the one present on teeth surfaces. With regard to clinical behavior, three clinical trials failed to highlight differences in clinical behavior between methacrylate and silorane-based composites.(Baracco, Perdigao,

Cabrera, & Ceballos, 2013; Mahmoud, Ali, & Hegazi, 2014; Yazici, Ustunkol, Ozgunaltay, & Dayangac, 2014) In these studies no restoration failed for secondary caries.

Flexural strength

Flexural strength was investigated to assess the possible influences of light-curing time and light-curing intensity on the mechanical properties of the tested materials but also to investigate if the influence of these parameters was similar on both mechanical and biological performances. According to ISO 4049/2014(Standard, 2000) specifications, dental restorative materials should have flexural strength values above 80 MPa.(Standard, 2000) Both the tested materials respect this standard even if silorane-based composite values were inferior to the tested methacrylate-based composite as already pointed out by another study.(Lien & Vandewalle, 2010) This conclusion can validate results from a recent clinical trial study in which most of the failures of silorane-based composite were due to fracture.[42] However, as shown by Goracci and others(Goracci et al., 2014), other conventional composites have flexural strength values similar to those of silorane-based composite.

ISO specifications also required the length of the specimens to be 21 mm. While this method may prove useful to provide completely polymerized specimens, it may not provide accurate information regarding the influence of light-curing parameters on specimens flexural strength, due to an overlapping

of the tip of the light-curing-source during polymerization. For this reason, the length of the bars (10 mm) differed from ISO specifications and was specifically chosen (as equal to the diameter of the fiberglass tip of the light-cure unit) in order to allow a single-shot polymerization of the specimens.

Results showed that composite type was the only significant factor ($p<0.0318$) while light-curing time and light-curing intensity did not influence flexural strength. Consequently, also the second null-hypothesis could be rejected.

No threshold value indicating a decrease in mechanical properties was identified for any of the tested light-curing times or light-curing intensities. Even if the manufacturer suggested a polymerization time of 20 s, testing after 24 h from light-curing process showed that maximum flexural strength values were already reached at 10 s independently of the light-curing intensity tested.

2.4.8 Conclusions

Within the limits of this study, it is possible to conclude that silorane-based composite is less prone to *S. mutans* biofilm development compared to a widely used methacrylate-based composite. Moreover, silorane surface colonization does not seem to be influenced by factors as light-curing time and light-curing intensity. This may potentially reduce the occurrence of secondary caries thus improving the longevity of direct composite restorations.

Flexural strength was not influenced by light-curing time or light-curing intensity but proved to be significantly higher for the methacrylate-based

composite.

It is interesting to notice the different influence of light-curing parameters on composite mechanical and biological performances.

2.5 Introduction

2.5.1 Polyether-ether-ketone (PEEK) surfaces influence *Streptococcus mutans* biofilm formation

Dental implants are often the first choice treatment for missing teeth replacement in modern Dentistry. While their 90% survival at 10 years (Bumgardner, Adatrow, Haggard, & Norowski, 2011) can't be considered inadequate, this achievement should be furtherly improved by reducing the incidence of periimplantitis, the main cause of implant failure over time. (Mouhyi, Dohan Ehrenfest, & Albrektsson, 2012) The presence of a pathogenic biofilm is a pre-requisite of this condition. (Kotsakis et al., 2016) In order to achieve better performances and better interactions with both the host and the biofilm permanently colonizing it, new materials and surface treatments should be developed. Among the materials recently introduced in Dentistry, polyether-ether-ketone (PEEK) is a polymeric material (Fuhrmann et al., 2014)that has been used in orthopedics for several years. This material has two advantages over other prosthetic materials such as titanium, it has a Young's modulus similar to the human bone and it can be easily additioned with other materials.(Wang et al., 2015) Nevertheless, the osteoconductive properties of

unmodified PEEK are limited when compared to those of titanium.(Najeeb, Bds, Bds, & Bds, 2016) In order to overcome this issue, several methods have been tested, including hydroxyapatite coatings and an increase in surface roughness.(Ma, Weng, Bao, Song, & Zhang, 2013) It must be pointed out, however, that several studies didn't highlight significant differences in osteointegration between modified PEEK, titanium and zirconia.(Najeeb et al., 2016)

Currently, there is a lack of knowledge on biofilm formation on unmodified-PEEK surfaces, with just a few literature up to date and no experimental results about biofilm formation on unmodified-PEEK surfaces using a drip-flow reactor (MDFR).

2.5.2 Aim of the study

The aim of this study was to evaluate *in vitro* the effect of PEEK surfaces and those of other commonly used restorative and prosthetic dental materials (resin-based composite, biomedical-grade stainless steel and titanium) on biofilm formation using a MDFR.

2.5.3 Materials and methods

Specimen preparation

PEEK, grade-V titanium, AISI-316L stainless steel, resin-based composite (RBC, control) and tissue-culture treated polystyrene (reference material)

where chosen as materials to be tested. Disk-shaped specimens for each material were made with a diameter of 6 mm and 1 mm height. A total of 15 specimens per group were produced.

RBC disks were obtained from a nanohybrid resin-based composite (RBC, Clearfil Majesty ES-2, shade A2, Kuraray Europe GmbH, Hattersheim am Main, Germany) by packing an excess of uncured material into a custom-made PTFE mold. The top and bottom surfaces of the RBC were covered with a cellulose acetate strip (Mylar) and condensed against a glass plate by centrally applying a load of 1 kg for 20 s. The specimens were then irradiated for 40 s (four consecutive cycles of 10s) by placing the tip of a hand-held light-curing unit (MiniLED, Satelec, Acteon Group, Merignac, France, 420-480 nm emission, 1,250mW/cm² light intensity) into direct contact with the acetate strip. All RBC specimens were then stored under light-proof conditions in distilled water for 6 days at 37 ± 1 °C. In order to minimize the impact of residual monomer leakage on cell viability, the distilled water was rinsed and replaced twice a day during that period.

Saliva preparation

Human saliva was collected according to the protocol of Guggenheim et al. (Guggenheim et al., 2001), as follows. Unstimulated whole saliva was collected from 5 healthy donors after obtaining written, informed consent and pooled. Saliva was clarified by centrifugation at 12000 rpm for 15 min at 4 °C, then

stored at -20°C until use.

Bacteria

Culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, U.SA) and reagents were obtained by Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, U.S.A.). Mitis Salivarius Bacitracin agar (MSB agar) plates were inoculated with *S. mutans* (ATCC 35668) and incubated at 37°C for 48 h in a 5% CO_2 supplemented environment. A pure culture of the microorganism in Brain Heart Infusion broth (BHI) was obtained from these plates after incubating at 37°C for 12 h in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (2,200 rpm, 19°C, 5 min), washed twice with sterile PBS and resuspended in the same buffer. The cell suspension was subsequently subjected to sonication (Sonifier model B-150; Branson, Danbury, CT, USA; operating at 7W energy output for 30 s) in order to disperse bacterial chains. Finally, the suspension was adjusted to a McFarland scale 1.0 optical density, corresponding to a concentration of approximately 3.0×10^8 cells/mL.

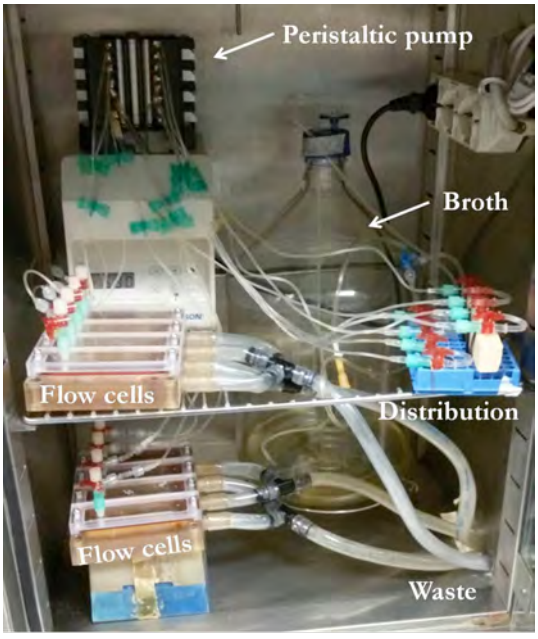
MDFR model

The specimens, previously sterilized using a chemical peroxide-ion plasma sterilizer (Sterrad; ASP, Irvine, CA, USA) were randomly divided into the four flow cells of the MDFR (**Fig.s 16-17**) and then incubated at 37°C for 24 h in

clarified saliva. The MDFR used in the present study was a modified version of a commercially available drip-flow reactor (DFR 110, BioSurface Technologies; Bozeman, MT, USA) (Goeres et al., 2009) (MDFR) according to a previous protocol.(Ionescu et al., 2015) The modified design allowed the placement of customized PTFE trays on the bottom of the flow cells to maintain the specimen surfaces into the flowing medium. To avoid microbial contamination, the tubing and the specimen-containing trays were sterilized with a chemical peroxide-ion plasma sterilizer (Sterrad; ASP, Irvine, CA, USA). The MDFR was then assembled inside a sterile hood and transferred into a thermostat operating at 37°C.

Each cell was then inoculated with 10 ml of bacterial suspension in early log phase to allow bacterial adhesion. After 4 hours, using a multichannel

Fig. 16
The modified drip-flow reactor inside the thermostat.



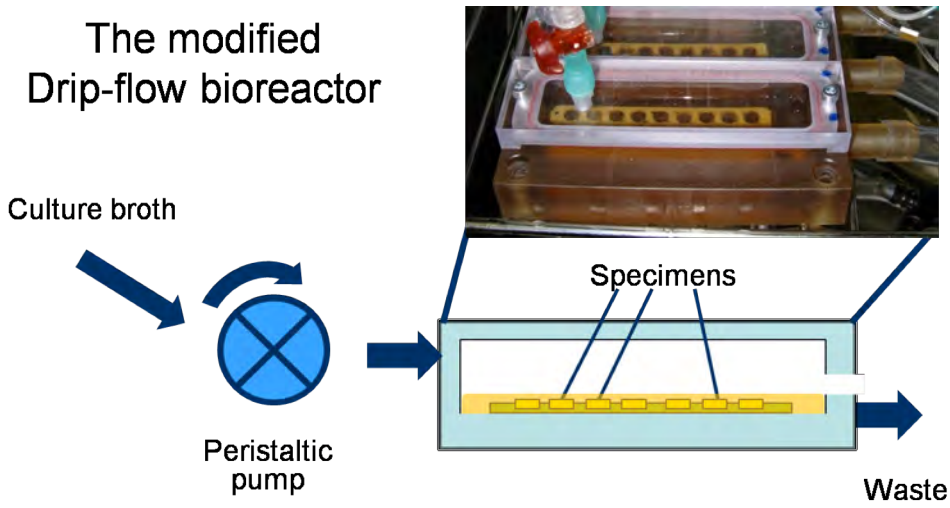
computer-controlled peristaltic pump (RP-1k; Rainin, Emeryville, CA, USA) a constant medium flow through the four flow cells was obtained. The nutrient medium composition was the following (Ionescu et al., 2012): 10.0 g/L sucrose, 2.5 g/L mucin (type II, porcine gastric), 2.0 g/L bacteriological peptone, 2.0 g/L tryptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L CaCl2, 0.1 g/L cysteine hydrochloride, 0.001 g/L hemin, and 0.0002 g/L vitamin K1. The flow-rate through each cell was set to 9.6 ml/hour. All samples were tested for biofilm formation 24 hours after flow start since the aim of the study was to evaluate the effect of different materials on a mature biofilm. After the incubation, the

Fig. 17

How the modified drip-flow reactor works:

The broth is pumped into the flow-cells and it covers the specimens inserted into the trays.

Bacteria can adhere and grow over the exposed areas of the specimens.



amount of viable biomass adherent to the samples was evaluated with the MTT assay as described in previous studies. (Brambilla et al., 2013; Hahnel et al., 2014)

SEM analysis

A total of two specimens for each tested material underwent scanning electron microscopy analysis. Specimens were sputter-coated with gold (JEOL FFC-1100; Tokyo, Japan). Images were taken at different magnifications (100x-10000x) using SEM (JSM 5300; JEOL, Inc., Peabody, MA) assessing surface

characteristics which may be related to biofilm formation.

Statistical analysis

All statistical analyses were performed using statistical software (JMP Pro 12.0, SAS Institute, Inc, Cary, NC, USA). The OD data are reported throughout the text as means and standard deviations calculated from the natural values. Normality of distributions was preliminarily checked using Shapiro-Wilk test and homogeneity of variances was preliminarily checked using Bartlett's test. A one-way ANOVA model was used with Tukey as post-hoc test to highlight significant differences ($p<0.05$).

2.5.4 Results

All results are displayed in **Fig. 18** as mean OD values (±1 standard deviation) which are directly proportional to the amount of viable, adherent biomass on the surfaces of the specimens. Each material showed higher biofilm formation when compared to the polystyrene used as control ($p=0.0018$). No differences among all the other tested materials were found.

SEM analysis is represented in Panel 1 as 5000x magnifications of representative fields of each tested surface.

PEEK surfaces (1A) appear very rough at a microscopic level, due to the machining procedures of the material. Machining procedures left grooves and irregularly distributed debris on titanium and steel materials (1B and 1C,

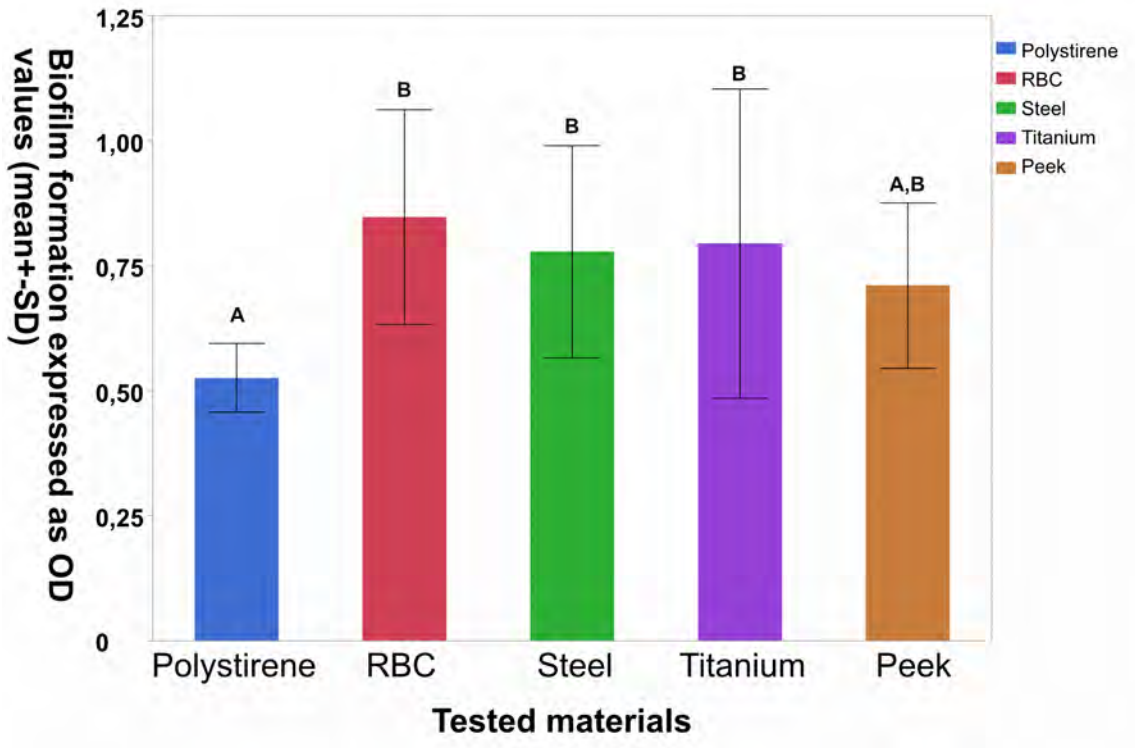
respectively). In particular, steel disks showed in some points the presence on the surface of nucleation sites with cristallites and nanospheres measuring 80-100 nm (1D).

Both control and reference materials (RBC and polystyrene, 1E and 1F, respectively) showed a very regular surface. RBC surfaces show an inhomogeneous appearance at a micro-level due to the presence of the nano-fillers.

Fig. 18

Results from the MDFR model after 24h incubation with *S. mutans* biofilm.

Different superscript letters stand for statistically significant differences.



2.5.5 Discussion and conclusions

The protocol considered a *S. mutans* biofilm model developed under continuous flow conditions inside the MDFR for 24h. PEEK was hence compared with different materials widely used in dentistry; polystyrene specimens were used as reference.

Data analysis allowed two main observations. Firstly, even in absence of significant differences between groups, PEEK seemed to be less colonized than the other tested materials. In **Fig. 18** data showed that the effect of the tested materials on biofilm formation was non-existent, with the exception of the reference material on which biofilm formation was slightly lower ($p=0.0018$). The MDFR run was repeated three times obtaining the same results every time.

A similar study was conducted in 2015 analyzing PEEK, titanium, zirconia and PMMA, the results showed that at 20h PEEK presented lower biofilm formation when compared to the other materials. (Hahnel, Wieser, Lang, & Rosentritt, 2015) Although being similar to our study, no MDFR was used in that case and multispecies biofilm formation was evaluated. This could justify the differences between the studies.

The second observation is that the SD of all experimental groups was very high except for polystyrene group. The high SD masked the possible presence of significant differences in biofilm formation between the tested groups, and this means that the standardization of experimental specimens surfaces should be improved in order to increase the significance of the results. This is supported

by the SEM observations of the specimens surfaces. Indeed, the presence of debris on steel and titanium, and the drawings, creepings and other surface alterations caused by the machining procedures on PEEK surfaces can account for the high SD shown by biofilm formation on these surfaces. On the other hand, it is well-known that composite materials show a high surface heterogeneity on a microscopic level due to the presence of filler-rich and resin-rich areas. (Ionescu et al., 2012) This may explain why RBC specimens had SD much higher than polystyrene, despite both possessed a very smooth surface.

It can be concluded that PEEK didn't show any antibacterial property. Several *in vitro* studies are necessary to corroborate the results of this preliminary study. Moreover, clinical *in situ* studies could be useful in order to evaluate this material in a more realistic, although less controlled, setting.

3 General discussion and conclusions:

In this PhD thesis the innovations in oral infectious diseases prevention and dental material development were investigated. The objective of this work was to use innovative solutions in order to reduce the prevalence of the most common infectious pathologies in the human kind: the oral diseases. Dentists keep considering the biofilm (dental plaque) as something to be necessarily eradicated in order to restore an healthy oral status. This generally works, but as it was demonstrated years ago, only a small fraction of the bacterial species

are in fact pathogenic and they become a problem when they are predominant. If the biofilm is eradicated with toothbrushes, both pathogenic and saprophyte species are eliminated. However, as recently confirmed, bacteria are essential for our health, furthermore, 90% of our genetic material is bacterial! We could not possibly live without them. To conclude, while eliminating oral biofilm is the easiest solution up to now in order to stop oral infectious diseases, a new approach is to influence the biofilm in order to keep the pathogenic species contained and promoting the saprophytic ones. As we saw in this thesis, among the solutions to induce a selective pression for saprophyte species there is our diet. In fact, cariogenic species use dietary carbohydrates and produce acids as metabolic products. Also, since these species are acid-resistant, the lowering of the pH sums up as a selective factor for them while disfavouring other species. However, not every carbohydrate is metabolized efficiently. It has been demonstrated that levorotatory ones and polyols are way less easy for the bacteria to digest, thus producing fewer acids.

Finally, replacing dextrorotatory carbohydrates with levorotatory ones and polyols in our diet may influence the biofilm in a positive way favoruing saprophytic bacteria instead of the pathogenic ones.

As for secondary prevention measures, the development of materials able to modulate the formation of biofilm is essential for the long term success of oral rehabilitations. Dental caries are generally treated with resin-based composite restorations but they are often subject to secondary caries. This could be

because these materials promote the growth of biofilm and it is possible that they also favours some cariogenic species. So, to overcome this issue new materials are introduced every year. Among them there are the siloranes, which are composite materials, developed to solve the polymerization shrinkage issue of the methacrylate-based composites but as we found out in our study, these materials also seem to be less susceptible of biofilm development.

Dental caries often leads to the impossibility of saving teeth which then need to be extracted and replaced. Titanium implants are probably the best treatment available up to now. However, they are subject to per-implantitis, and infectious disease causing an inflammatory response in the hosts which ultimately leads to the loss of bone support and finally to implant failure. To prevent this widespread problematic different strategies have been proposed, such as different surface treatments or the use of different materials. In this thesis we investigated the use of PEEK, a promising material for both implantology and prosthetic dentistry. It was concluded that it did not have any anti-biofilm properties, however, further studies will be done in order to assess if the species which grow on this material are mainly pathogenic or saprophyte.

The main limitation of the studies conducted for this thesis can be syntetized in the famous sentence “The whole is greater than the sum of its part”. Why? Because oral biofilms are very complex ecosystems while we examined single or double species biofilms. This approach was chosen to better understand the behavior of the main pathogenic species. To sum up, this is a limit but also a

point of strength of the thesis because it allowed us to exclude the influence of other unknown species from the results. In the future, further experiments will be performed in order to have a more complete view on this topic.

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