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WILLIAM CUSTODIO

Influência das proteínas salivares e plasmáticas no desenvolvimento de biofilmes de *Candida albicans*

Tese de Doutorado apresentada a Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do título de Doutor em Clínica Odontológica – Área de Prótese Dental.

Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

Este exemplar corresponde à versão final da Tese defendida pelo aluno William Custodio, e orientada pela Profa. Dra. Altair Antoninha Del Bel Cury.

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RESUMO

O desenvolvimento de biofilme de *Candida albicans* pode ser mediado pela expressão diferencial de sítios de ligação protéicos na película adquirida formada sobre as superfícies das próteses dentais. Assim, objetivo geral deste estudo foi verificar a influência das proteínas de origem salivar e plasmática na formação dos biofilmes de *C. albicans.* No primeiro capítulo foi revisado o estado da arte de metodologias aplicadas para análise de proteínas. A partir do conhecimento das metodologias, a pesquisa foi realizada com objetivo de caracterizar os perfis protéicos de películas adsorvidas (ADP) à superfície de espécimes de poli(metilmetacrilato), na presença de saliva ou saliva acrescida de plasma sanguíneo. A composição da ADP foi analisada utilizando a técnica de espectrometria de massas, cujo resultado demonstrou diferenças significativas nos proteomas obtidos entre os grupos. Ainda, foi verificada a influência destas películas na energia livre de superfície (SFE) e expressão de fatores de virulência de biofilmes de *C. albicans*. Os achados demonstraram que a ligação de proteínas plasmáticas determinou aumento de ambas variáveis. A partir desses dados estudou-se o efeito individual de algumas das proteínas identificadas na ADP no desenvolvimento de biofilmes de *C. albicans*. Com esse objetivo, biofilmes de *C. albicans* foram desenvolvidos (90 min, 24, 48 e 72 h) sobre películas mono protéicas de albumina, mucina I e II, lactoferrina, fibrinogênio, C3b, IgA e histatina 5. Em cada um dos tempos foi avaliada a atividade metabólica da célula fúngica (teste de XTT), a organização estrutural do biofilme (microscopia confocal por varredura à laser) e a expressão de fatores de virulência por meio dos testes de produção enzimática. Baseado nas evidências geradas por estes trabalhos, pode-se concluir que proteínas salivares e plasmáticas adsorvidas como integumentos protéicos mantêm sua função biológica e, assim, exercem uma influência modulatória no desenvolvimento de biofilmes de *C. albicans*.

Palavras-chave: Saliva; Plasma; Película adquirida; *C. albicans*; resina de PMMA.

ABSTRACT

The development of *Candida albicans* biofilms may be mediated by a differential expression of proteic ligand sites in the acquired pellicle formed onto the oral prosthesis surfaces. Therefore, the main objective of this study was to evaluate the influence of salivary and plasmatic-derived proteins on the development of *C. albicans* biofilms. In the first chapter the state-of-the-art was reviewed for methodologies applied to the analysis of proteins. From the knowledge of the methodologies, the research was realized aiming to characterize the protein profile of acquired pellicles (ADP) on poly(methylmetacrilate) surfaces' specimens in the presence of saliva or saliva supplemented with blood plasma. ADP composition was analyzed by mass spectrometry techniques, which results demonstrated a significant difference in the obtained proteome between the groups. Moreover, the influence of these pellicles was evaluated for the surface free energy (SFE) and expression of virulence factors by *C. albicans* biofilms. The findings demonstrated that the binding of plasmatic proteins in the ADP determined increases in both experimental variables. From these data, the individual effect of some identified ADP proteins was evaluated on *C. albicans* biofilm development. With this objective, *C. albicans* biofilms were developed (90 min, 24, 48 and 72 h) over mono-protein pellicles of albumin; mucin I and II; lactoferrin; fibrinogen; C3b; IgA and histatin 5. For each time point, biofilms were analyzed for metabolic activity (XTT assay), structural biofilm conformation (Confocal scanning laser microscopy) and, expression of virulence factors by means of enzymatic activity assays. Based on the generated evidences of these studies, we can conclude that adsorbed salivary and plasmatic proteins as proteic integuments keep their biological function, and thus exert a modulatory influence on *C. albicans* biofilm development.

Key Words: Saliva; Plasma; Acquired pellicle; *C. albicans*; PMMA resin.

SUMÁRIO

INTRODUÇÃO

O fenômeno da inversão da pirâmide populacional, devido ao aumento da expectativa e qualidade de vida, contribui para o aumento do número de pacientes reabilitados com próteses dentais removíveis e/ou implanto suportadas (Fueki *et al.,* 2007; Sachdeo *et al*., 2008). Nestas reabilitações, a resina à base de poli(metilmetacrilato) tem sido um dos materiais odontológicos mais amplamente utilizados. Em que pese as boas qualidades estéticas desse material, devido suas características de superfície, quando as próteses são expostas ao ambiente oral, este substrato torna-se passível à colonização microbiana. Assim, as superfícies protéticas a base de PMMA são um nicho favorável à formação de biofilmes bacterianos e fúngicos, sendo o último definido como uma comunidade estruturada composta por leveduras, hifas, pseudo-hifas e uma matriz extracelular, permeada por uma fase líquida (Baillie & Douglas, 2000).

Aproximadamente 45,3% dos usuários de próteses dentais removíveis são acometidos por patologias fúngicas associadas ao desenvolvimento e expressão de fatores de virulência de biofilmes fúngicos (Budtz-Jorgensen *et al*., 1975). Embora presente de maneira comensal na microbiota oral (Davenport, 1970), entre os microorganismos relacionados à candidose oral associada ao uso de próteses, *Candida albicans* tem sido apontado como o microorganismo de maior prevalência e virulência (Chaffin *et al*., 1998; Nikawa *et al*., 1998; Darouiche, 1998; Dar-Odeh *et al*., 2003). Entre os fatores desencadeantes desta patologia oral relacionados à célula fúngica citam-se a hidrofobicidade da parede celular do microrganismo bem como a presença de sítios de ligação na mesma, cuja interação com a película adsorvida influencia a adesão inicial de células planctônicas (Gibbons *et al*., 1989; Scheie *et al*., 1994; Moura *et al*., 2006). Contudo, o estabelecimento do quadro patológico é dependente, além de fatores relacionados à célula fúngica, a fatores relacionados ao hospedeiro, como o ambiente oral, imunossupressão, higienização deficiente da prótese dental, uso de fármacos de forma profilática, entre outros (Cannon *et al*., 1999).

Estudos prévios demonstraram que alterações na cavidade bucal como variações do conteúdo protéico nos fluídos orais, podem modular positivamente a virulência de biofilmes orais (Samaranayake *et al*., 1980; Rotrosen *et al*., 1986). Neste contexto, a candidose oral associada ao uso de próteses, pode alterar a disponibilidade de proteínas plasmáticas no ambiente oral, pois nos achados clínicos essa patologia caracteriza-se por inflamação da mucosa da área chapeável, eritema e recorrência de micro-traumas da mucosa oral (Budtz-Jorgensen, 2000). Contudo, trabalhos apontando a influência de proteínas salivares e plasmáticas adsorvidas ao substrato de poli(metilmetacrilato) principalmente no que diz respeito às proteínas plasmáticas e a expressão dos fatores de virulência de *C. albicans* são escassos (Chaffin *et al*., 1998; Xu *et al*., 2008). Embora a caracterização da composição protéica e as propriedades de superfície da película adsorvida sejam essenciais para o entendimento fisiopatológico da candidose associada ao uso de próteses, pouco se sabe sobre o perfil protéico de películas adsorvidas à superfície de PMMA.

Neste contexto, o fenômeno da adesão das células de *C. albicans* é desempenhado por proteínas especializadas da superfície celular chamadas de adesinas que se ligam especificamente a aminoácidos ou carboidratos presentes na superfície de outras células ou em superfícies abióticas (Verstrepen & Klis, 2006). Estudos prévios demonstram que compostos protéicos presentes na película adsorvida, oriundos da saliva e do plasma sanguíneo, como mucinas (tipo I e II), imunoglobulinas (IgA e IgG), histatinas (van Nieuw Amerongen, 2004), fibrinogênio, fatores do sistema complemento (Calderone *et al*., 1991) e lactoferrina (Viejo-Díaz *et al*., 2004) interagem na adesão da célula fúngica ao prover sítios de interação ou mesmo por servir como barreira para a agregação do microorganismo ao substrato. Contudo, a adsorção de proteínas ao substrato é um processo seletivo que resulta em interações moleculares dinâmicas, comportamento em parte assegurado pela maior participação de ligações não covalentes entre as proteínas e o substrato, resultando na expressão diferencial de sítios de ligação protéica (Gocke *et al*., 2002). Além do perfil químico das

interações estabelecidas, as propriedades mecânicas destas, como força de adesão, propriedades friccionais e módulo de elasticidade, podem relacionar-se com a disponibilidade e perpetuação de sítios de ligação protéica e conseqüentemente à colonização de microorganismos como a *C. albicans*, passo primeiro para a estomatite protética. Esta afirmação é evidenciada por mecanismos moleculares, como o observado para proteases inibidoras cuja forte ligação correlaciona-se diretamente com sua atividade na saliva, resultando na maior penetração de hifas da célula fúngica no tecido do hospedeiro (Gocke *et al*., 2002).

Ainda relacionado à patogenicidade de *C. albicans*, a transição de levedura para hifa é considerada um importante fator de virulência (Cutler, 1991), por ocorrer em resposta a diferentes fatores ambientais como, por exemplo, composição dos fluidos orais (Simonetti *et al*., 1974; Buffo *et al*., 1984, Chaffin *et al*., 1998; Nikawa *et al*., 1998; Csank *et al*., 2000). Como resultado da conversão morfo-genética, enzimas hidrolíticas são codificadas e secretadas, acarretando em irritação da parede celular epitelial da barreira mucosa (Chaffin *et al*., 1998; Kumamoto & Vinces, 2005; Xu *et al*., 2008). Dessa maneira, películas adsorvidas com diferentes perfis protéicos, em resposta às variações dos fluidos orais, podem potencialmente modular a atividade proteolítica enzimática e conseqüentemente a perpetuação ou intensificação do processo inflamatório, gerando um ciclo vicioso (Nikawa *et al*., 1998). Portanto, o estabelecimento do "*fingerprint*" peptídico e a análise funcional dos constituintes orgânicos da película adquiridas sejam elas, mono ou multi-protéicas, podem fornecer dados importantes na validação de biomarcadores implicados nesta condição patológica, que em casos avançados e, em especial pacientes imunossuprimidos e/ou idosos frágeis, pode levar a candidemia cuja taxa de mortalidade é alta (Wey *et al*., 1988; Leleu *et al*., 2002; Cheng *et al*., 2005).

Neste contexto, o objetivo deste estudo foi identificar e avaliar o efeito de proteínas salivares e plasmáticas, no desenvolvimento de biofilmes de *C. albicans.* Com esse objetivo este trabalho foi divido em três capítulos. No capítulo I

foi apresentada uma breve visão translacional sobre os avanços tecnológicos, como espectrometria de massas e microscopia atômica, e suas aplicações a sistemas biológicos, como na identificação e caracterização funcional de proteínas salivares como fatores preditores às patologias orais, por exemplo, nas infecções fúngicas. O capítulo II discorre sobre a caracterização proteômica da película adquirida formada sobre a superfície de próteses dentais e a influência de seus componentes protéicos tanto no substrato, quanto no biofilme fúngico formado sobre o mesmo. No capítulo III é apresentado e discutido o impacto individual de proteínas da película adquirida no desenvolvimento de biofilmes de *C. albicans*, considerando a morfologia, bioatividade e virulência dos biofilmes. Os resultados gerados e discutidos neste trabalho poderão contribuir para a prevenção e tratamento da candidose associada ao uso de próteses, tendo como base o reequilíbrio fisiológico dos fluídos orais e o desenvolvimento de biomateriais para a base de próteses.

CAPÍTULO I

Salivary proteins as predictors and controls for oral health

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Abstract

We will provide a translational view of using the recent technological advances in dental research for predicting, monitoring, and preventing the development of oral diseases by investigating the diagnostic and therapeutic role of salivary proteins. New analytical state-of-the-art technologies such as mass spectrometry and atomic force microscopy have revolutionized the field of oral biology. These novel technologies open avenues for a comprehensive characterization of the salivary proteins followed by the evaluation of the physiological functions which could make possible in a near future the development of a new series of synthetic protein for therapeutic propose able to prevent global oral diseases such as periodontal disease and dental caries, the two most prevalent oral diseases in the World.

Keywords Saliva: Proteins, Proteomics, Oral disease, Mass spectrometry, Atomic Force Microscopy.

Introduction

Periodontal disease (i.e., gingivitis, chronic periodontitis) and dental caries are the two most globally prevalent chronic oral pathologies that affect children, youth, adults, and elders (Featherstone, 2000; Albandar, 2002). In the United States, gingivitis affects 50% of adults, while chronic periodontitis affects an estimated 35% of the adult population (Albandar *et al*., 1999). In addition, each year over 300,000 patients worldwide are diagnosed with oral cancer (Parkin *et al.,* 1988), representing 2–3% of all malignancies (Parkin *et al*., 2005). More than 90% of these cases are categorized as oral squamous cell carcinoma (SCC), with high metastasis rates, resulting in high patient mortality (Neville and Day 2002; Parkin *et al*., 2005).

Recent advances in dental research have enforced the need to gain a more comprehensive understanding of the prevention, treatment, and management of oral diseases. Oral health is an essential component of an individual's well-being because it is very closely related to general health. Throughout the years, oral diseases have been defined as localized oral disturbances, but recent research suggests that they can be considered as general health distal determinants, acting as comorbidities and risk factors for many systemic diseases. For instance, the association between diabetes mellitus and periodontal disease can be considered to be bidirectional: diabetes can be a risk factor for the development of periodontitis (diabetic patients are 2.1–3.0 times more at risk of developing periodontitis; Salvi *et al*., 1997), while patients with periodontitis are much more likely to develop diabetes (Grossi and Genco 1998; Deshpande *et al*., 2010). Considering the interconnectedness of oral diseases and general health, we must gain a complete understanding of the pathophysiology of oral diseases within the dynamic, complex oral cavity in order to successfully develop potential treatments and accurate patient risk assessments for the prediction of these diseases (i.e., biomarkers).

Periodontitis and dental caries are multi-factorial diseases primarily dependent on biofilm development. The oral cavity fosters an intricate microbial ecosystem, consisting of more than 700 bacterial species, many of which play an

important role in maintaining oral health (Aas *et al*., 2005). However, when this ecosystem becomes disrupted, an increase in pathogenic microorganisms occurs, resulting in the initiation of disease. To initialize the growth of pathogenic biofilms and therefore the development of oral disease, microbial adhesion to the oral surface, such as dental enamel or denture resin is the first and essential step to prevent cells from being removed by salivary flow (Whittaker *et al*., 1996; Jenkinson and Lamont, 1997). Human saliva plays a significant role in controlling microbial adhesion since its proteic components, after adsorbed to the oral surface, result in the formation of salivary protein pellicles.

The acquired pellicle (AP) is a protein integument formed on the oral surface immediately after exposure of saliva to the oral environment. This protein film formed on the dental enamel is a result of specific physical bonds (i.e., hydrophobic, hydrogen bonding, ionic, and van der Waals bonds) between the substratum surface and the salivary molecules (i.e., salivary proteins, peptides, carbohydrates, lipids; Dawes *et al*., 1963; Rolla *et al*., 1983; Siqueira *et al*., 2007a), resulting in the development of a 100–1000 nm protein film on the oral surface for microorganisms to adhere (Kuboki *et al*., 1987; Skjorland *et al*., 1995).

The AP has important binding sites for oral microbiota; the protein-microbial adhesion process involves stero-specific interaction between receptors on the pellicles and adhesions on the microbial cell surfaces (Scannapieco *et al*., 1994). The AP may control the adhesion of pathogenic microbes to oral surfaces because some salivary pellicle proteins found in vivo can inhibit or enhance growth of oral microbiota (Scannapieco *et al*., 1994). For instance, the carboxylterminal of histatin 5 demonstrates potent fungistatic and fungicidal effects against pathogenic fungi, *C. albicans*, at concentrations found in salivary secretions of healthy individuals (15–30μM) (Oppenheim *et al*., 1986; Xu *et al*., 1991). The antimicrobial affect of histatin 5 is due to its composition of multiple basic amino acid residues (arginine and lysine), allowing this salivary protein to possess a basic character capable of disrupting the cell membrane by forming membrane pores, inducing membrane

permeability (increased loss of K^+ from cell) and resulting in cell death (Pollock *et al*., 1984).

In contrast, the carboxyl-terminal of acidic proline-rich proteins (PRPs) promotes the attachment of various oral bacteria (i.e., Streptococcus and Actinomyces sp.) to the AEP, thus enhancing microbial colonization of the tooth surface (Gibbons and Hay, 1988). Specifically, the ProGln terminal of acidic PRPs is the preferred protein-binding site for microorganisms including *S. gordonii* (Gibbons *et al*., 1991). Similarly to acidic PRPs, the carboxyl-terminal of statherin binds a variety of potentially invasive oral microbiota, including *P. gingivalis* (Amano *et al*., 1994) and *C. albicans* (Cannon *et al*., 1995). In addition, at concentrations of 100 µg/mL (healthy individuals), statherin is capable of inducing the transition from virulent, hyphael *C. albicans* to the cocci form (Leito *et al*., 2009).

Recent studies have shown that pathogenic microorganisms have increased their resistance to natural host defenses and to antimicrobial treatments, resulting in more persistent and serious infections (Ramage *et al*., 2006; Tsang *et al*., 2007). This enforces the need for the development of novel antimicrobial treatments that would inhibit and/or kill pathogenic microbes, preventing further colonization and development of oral diseases. Since certain salivary proteins affect the growth of pathogenic oral microbes, their potential role in treatment/prevention of oral diseases must be considered.

Challenges

In order to evaluate the effectiveness of antimicrobial salivary proteins as a potential novel therapeutical approach for the combat of oral diseases, it is important to gain a comprehensive understanding of the inhibitory effects salivary proteins exhibit on pathogenic oral microbiota. One approach is to design larger-scale reaction systems that can allow us to control variables of interest (i.e., microbial consortia) and target specific questions about salivary protein-microbial interactions. Throughout the years, many in vitro model systems that model the

oral cavity have been designed involving either flow cells (Christersson *et al*., 1987; Larsen and Fiehn, 1995; Guggenheim *et al*., 2001) and even chemostats (Herles *et al*., 1994; Bradshaw *et al*., 1996; Kinniment *et al*., 1996; Bowden, 1999). However, some of these models yield contradictory results due to the selection of different parameters. Since the oral cavity is an extremely complex and dynamic system, many different components need to be considered when designing these systems, including multi-species biofilms, flow rate, temperature, pH, nutrient fluxes, and choice of proteins. Considering that human saliva consists of 2290 proteins (Loo *et al*., 2010) and 130 proteins in the AP (Siqueira *et al*., 2007b) it becomes incredibly challenging to reproduce the in vivo environment.

Another challenge when investigating the role of salivary proteins on oral biofilms is being able to view the world of a microbe on a small, micro-meter-scale. The advancements in high-resolution microscopy instruments have facilitated the investigation of microbial interactions (i.e., scanning electron microscopy, confocal microscopy, transmission electron microscopy). In addition to these tools, atomic force microscopy (AFM) has revolutionized the field of oral microbiology, enabling us to make a variety of protein/cell surface measurements on the atomic magnitude, directly in aqueous solution. Unlike conventional microscopy, AFM allows us to study adhesive (Lodish *et al*., 2004), mechanical (Greenleaf *et al*., 2007), electrostatic (Barkai *et al*., 2004), and immunochemical (Horber and Miles, 2003) nanoscale-level properties. In order to successfully conduct these measurements, the AFM cantilever-tip is typically functionalized with the protein/cell of interest and then used to probe a substrate (Zhang *et al*., 2009). However, the attachment of a pre-functionalized microsphere to the cantilever provides a much higher surface area when probing the substrate of interest, therefore greatly expanding the spectrum of adhesive interactions that can be obtained by a single cantilever (Ounkomol *et al*., 2009). For instance, streptavidin-coated microspheres can be attached to the AFM cantilever-tip (Fig. 1), and then reacted with biotinylated protein of interest to obtain an AFM functionalized, high-surface area probe (Zhang *et al*., 2009). This novel AFM-based force spectroscopy

approach allows us to determine biophysical properties of cells and proteins, and also enables us to measure single cell-protein adhesion interactions; the first pathophysiologic phenomena that occurs prior to the development of biofilms on oral surfaces. By manipulating microbial-protein interactions, we could essentially control oral disease development at the pellicle level, to prevent initial microbial adherence that leads to the development of oral diseases.

In addition to the use of these microscopic techniques, the development of revolutionary mass spectrometry has allowed for the investigation of microbial-protein interactions using a proteomic approach, allowing us to understand how salivary proteins affect metabolic pathways inside a cell. Moreover, the analysis of the composition of the AP can lead to the establishment of biomarkers and therefore the prevention of oral diseases that can impact general health. These powerful microscopic and proteomic techniques should be combined when investigating protein-microbial interactions in order to obtain a representative and comprehensive understanding of microbial responses to antimicrobial agents.

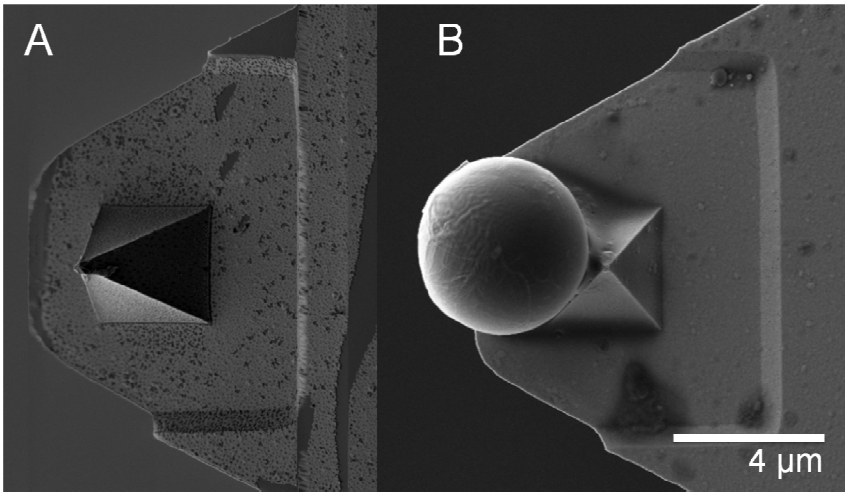

Fig. 1 Scanning electron micrograph of AFM cantilever (a) and of streptavidin-coated silica microsphere (~5 µm) adhered to AFM cantilever-tip via Araldite® epoxy glue (b). This functionalized microsphere can be coated with biotinylated proteins of interest to create an effective AFM probe for protein-adhesion measurements.

Salivary components as diagnostic tools for oral diseases

The advancement of new technology and instrumentation will enable us to obtain reliable protein fingerprints based on saliva and/or the acquired pellicle. Combining this information with a patient's oral microbiome can provide health care professionals with a comprehensive grasp of each patient's pathophysiological state.

A patient's saliva sample could potentially have individual proteins or groups of proteins that could be powerful biomarkers for oral diseases, including oral cancer. This is because saliva contains secretions from gingival crevicular fluid along with major and minor salivary glands, and is much less invasive compared to blood sampling (Spielmann and Wong, 2011; Edgar, 1992; Siqueira and Dawes, 2011). For example, it has been demonstrated that patients diagnosed with head and neck squamous cell carcinoma exhibit elevated levels of soluble CD44 (solCD44), a family of salivary isoforms, compared to cancer-free patients (Franzmann *et al*., 2007). In addition, patients with early childhood caries exhibit higher levels of glycoprotein, while caries-free patients demonstrate elevated amounts of proline-rich protein in saliva (Bhalla *et al*., 2010). Therefore, analyzing the protein composition of a patient's saliva could provide a protein profile for that patient, which could contain information as to which salivary proteins are up regulated/down regulated. This information could ultimately be compared to protein profiles of healthy patients, and those with different stages of various oral diseases, in order to accurately diagnose a patient (Blicharz *et al*., 2009).

When considering oral diseases that initiate on hard surfaces (i.e., dental caries), sampling the acquired pellicle and obtaining corresponding protein profile can likely be much more important compared to saliva (Siqueira *et al*., 2007a; Siqueira and Oppenheim, 2009). Pellicle formation is a highly selective process since only a fraction of proteins found in human saliva (130/2290 proteins) are present in the in vivo AP on dental enamel surface (Siqueira *et al*., 2007b). Since more than 51% of the recently identified pellicle proteins have unknown biological functions (Siqueira *et al*., 2007b), future research should focus on identifying the

biological function of the remainder of the pellicle proteins. There may in fact be additional proteins, or protein complexes, that could be even stronger biomarkers/predictors for various oral diseases.

In addition to obtaining saliva and AP protein patient profiles, sampling the oral microbial community (microbes adhered to oral surfaces) could produce a microbial patient profile, which could also be used as a biomarker for oral disease. Certain oral microbes adhered to the pellicle become more prevalent in patients suffering from certain oral diseases. For instance, patients exhibiting dental caries have an oral microbiota dominated by acidogenic and acid tolerant gram-positive bacteria (i.e., Streptococcus and Lactobacilli sp) (Marsh, 2003). Meanwhile, patients with periodontal disease have an increased proportion of obligately anaerobic bacteria (i.e., gram-negative species) (Socransky *et al*., 1998). The presence/absence or quantity of certain individual microbes, or even microbial community composition, adhered to the pellicle can correspond to various stages of oral disease development. Health care professionals could employ techniques such as the Human Microbial Identification Microarray to determine the microbial community of the oral cavity, and determine 'predictor' microbes of oral diseases.

Future direction

Gaining a comprehensive understanding of interactions between oral microbiota and salivary antimicrobial proteins could potentially result in the development of novel treatments for a variety of oral pathologies. It would be very interesting if a potential treatment for oral diseases could be currently in our oral cavity in the form of salivary proteins. Therefore, biofilm-dependent oral diseases can be controlled at the pellicle level the interface between pathogenic microbes and the solid oral surfaces. By controlling, or perhaps altering, the composition of the pellicle, we could potentially interfere the adhesion process of the oral microbiota to oral surfaces. Therefore, the future of oral therapeutics should focus on the interaction between salivary proteins and microorganisms.

In addition, the oral cavity contains biomarkers for oral diseases hidden within its complex oral fluids, AP integuments, and microbial consortia. The use of these proposed salivary biomarkers would promote health professionals to change their focus from disease diagnosis to monitoring and detecting oral disease at onset. Longitudinal studies should be conducted to better understand if such biomarkers could be used to identify progression of oral diseases. Ultimately, multiple biomarkers should be combined to achieve optimum specificity and sensitivity for detection of oral diseases.

Conclusion

Saliva is a complex fluid that possesses many important functions that relate directly to oral health. Accurate analysis of salivary components is a relatively new tool for assessing biological markers (hormones, immunoglobulins and antimicrobial proteins) for oral diseases as dental caries, periodontitis and oral candidiasis. The fingerprint profile of immunological compounds, such as immunoglobulin and other antimicrobial proteins in saliva samples can be an indicator of the host immune system's stress response to acute systemic disturbances, whereas assessment of the pellicle salivary constituents can identify susceptibility to local infections. Assessing proteins physical properties (i.e., adhesion forces) on different surfaces, cells or even to other proteins (protein-protein complexes) would assist us in understanding the role of salivary proteins and the pathophysiology of oral diseases. Subsequently, this new knowledge would help in developing innovative and effective therapeutics approaches to maximize the prevention of pathologic biofilm development. In conclusion, assessing and understating salivary composition can be applied as a feasible and reliable tool for predicting and treating several oral infections, diagnosing systemic diseases and determining the state of patient's immune systems. Therefore, collecting and analyzing saliva would not only help to better monitor and maintain the oral health of patients, but it could also significantly improve the health care system.

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CAPÍTULO II

Acquired denture pellicle: proteomic and functional characterization

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Abstract

The acquired denture pellicle (ADP) is assumed to play a role in *C. albicans* biofilms development. However, the presence of blood plasma proteins can modify it. This study evaluated the ADP composition formed on poly(methylmethacrylate) resin in the presence of whole-saliva (WS) or WS supplemented with blood plasma (WSBP) and, its effects on the surface free energy (SFE) and expression of virulence factors by *Candida albicans* biofilms. ADP proteome was analyzed by mass spectrometry coupled with liquid chromatography (LC-MS/MS). SFE was determined by acid-base technique. Phospholipase and proteinase enzymatic assays were evaluated in biofilms of *C. albicans* at 1.5, 24, 48 and 72h. Data were analyzed by two-way ANOVA and Tukey test (P=0.05). WS-derived ADP proteins included mostly α-amylase, cystatins, mucins and host-immune system proteins. In contrast, proteins related to the transport of other molecules were observed in WSBP-ADP i.e., fibrinogen and albumin. Compared to the WS-derived ADP, WSBP-derived ADP increased the SFE ($p<.05$) and the expression of *C. albicans* virulence factors ($p<.001$), regardless of the biofilm stage of development. In conclusion, the differential proteome of WSBP-derived ADP creates a hydrophilic surface and triggers *C. albicans* virulence.

Keywords: Polymethyl Methacrylate; Salivary Acquired Pellicle; Mass Spectrometry; Surface Properties; *Candida albicans*, Virulence Factors.

Introduction

Selective interactions between complementary molecules in the external part of the fungal cell wall and the proteic content of the acquired denture pellicle (ADP), a protein integument formed onto the dentures surface, may play a significant role in the pathogenesis of *Candida*-associated denture stomatitis. However, low sensitivity investigative approaches applied in the ADP proteome characterization and the high influence exerted by the oral environment lead data on this issue rare and controversial (Göcke *et al*., 2002; Karkowska-Kuleta *et al*., 2009).

Early investigations regarding ADP composition suggested that during *Candida*-associated denture stomatitis the oral prosthesis surface is highly exposed to blood plasma (Edgerton and Levine, 1992; Barbeau *et al*., 2003). Based on the evidences that some plasma proteins have the ability to cause displacement of precursor adsorbed neighboring proteins (Vroman, 2008), one hypothesis is that plasma proteins potentially determines the final ADP composition and, subsequently, the modulation of *Candida* infection and virulence. In addition it has been proposed that the binding of proteins with different polarities onto a substratum may alter its physicochemical surface properties, which have a significant effect on *C. albicans* colonization (Park *et al*., 2003).

Therefore, this study aimed to characterize the proteome of a salivary-blood plasma derived ADP and its influence on the substratum surface free energy and on the expression of *C. albicans* virulence.

Material and Methods

Study design

This randomized and blinded *in vitro* experiment was conducted with WS and WSBP pellicles as study factors. The variables were ADP proteome, SFE and the hydrolytic enzymatic activity of *C. albicans* biofilms. Poly(methylmethacrylate) resin discs with standardized surface roughness were used as substratum and the number of specimens used in each assay was based on previous literature (Price

et al., 1982; Combe *et al*., 2004). In order to avoid protein or cellular aggregation due gravity forces, metallic holders were used to maintain the specimens in the vertical position during pellicle formation and biofilm development. All experimental assays were conducted in triplicate on four separate occasions.

Preparation of PMMA substrate

Poly(methylmethacrylate) discs were fabricated according to the manufacturers' instructions. After the polymerization cycle (water bath/100°C/20 minutes) resin specimens (10 mm in diameter by 2 mm in thickness) were immersed in purified water (48 h/37°C) for the release of residual monomer (Pereira-Cenci *et al*., 2007). In order to simulate the inner side of a denture and to standardize the surface roughness in 0.31 ± 0.01 µm, the specimens surfaces were grounded in a horizontal polisher (model APL-4; Arotec, Sao Paulo, Brazil) by using progressively smoother aluminum oxide papers (320, 400, and 600 grit). The surface roughness was measured by a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) with 0.01 mm of accuracy. Subsequently, the specimens were attached to metallic holders and subjected to disinfection with 70% alcohol and ultrasonically cleanse with purified sterile water for 20 min in order to remove any contaminants and residues.

Acquired denture pellicle formation

A pool of stimulated whole saliva (WS) from four healthy subjects, 2 male and 2 female, with a mean age of 26.5 yrs, was used for the experiments. The exclusion criteria's applied included presence of periodontal disease, mucosal lesions, erythema, smoking habits, history of immunodeficiency or autoimmune disorders and the use of medications known to affect salivary production for at least 15 days before the collection day. Informed written consent, in agreement to the Ethical Committee of the Piracicaba dental School FOP-UNICAMP-BR, was obtained from each patient.

Immediately prior to the experimental tests, whole saliva production was stimulated by mastication of Parafilm M and collected in ice-chilled polypropylene tube (approximately 25 mL/donor). Saliva collection was done in the morning, from 7:00 am to 8:00 am, during 30 min. Salivary samples were then mixed, clarified by centrifugation (10.000 *g*/5 min/4°C), filtered through a 0.22 µm membrane filter (Corning, NY, USA) and treated with 0.1 mM EDTA and 0.1 mM PMSF. In order to develop the WSBP-derived ADP, human blood plasma from 4 health individuals was mixed and dispensed in a 20 mL tubes containing the processed WS in a ratio of 1:95 (µg/µg). Therefore, the discs were placed in a pre-sterilized flat bottomed 24-well tissue culture plates and incubated with 2 mL of WS (control group) or WSBP (experimental) solutions (2 h/37°C/75 rpm). Afterwards, the specimens were gently washed three times with phosphate buffered saline (PBS) pH 7.4 to remove unbound proteins components from the adsorbed pellicles and then, immediately used in the experimental assays.

ADP extraction and mass spectrometry analysis

ADPs were extracted by the method described previously by Yao *et al.,* 2003, with minor modifications. Briefly, the resin discs were put together in a polypropylene tube and then vortexed (30 s) in 20 mL of purified water followed by sonication (7 watts/4°C) over a five-minute interval. Total protein concentration was quantified by the Bradford method (Bradford, 1976). The extraction procedure resulted in a mean protein concentration of 0.07 µg/µL, for both groups.

ADP samples (15 µg) were reduced, alkylated and digested with trypsin (1:50, w/w) (Paes Leme *et al*., 2011). The resulting peptides were ressuspended in 12 µL of 0.1% formic acid and than separated by C18 (100 µm x 100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-Tof Ultima mass spectrometer (Waters) with nanoelectrospray source at a flow rate of 0.6 µL/min. The gradient was 2-90% acetonitrile in 0.1% formic acid over 60 min for the digested proteins. The nanoelectrospray voltage was set to 3.5 kV, a cone voltage of 30 V and the source temperature was 100°C. The instrument was operated in the 'top three'

mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on exclusion list for 60s.

The spectra were acquired using software MassLynx v.4.1 and the raw data files were converted to a peak list format (mgf) by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ldt.) and searched against Human International Protein Database (IPI) v.3.72 (86392 sequences; 35093930 residues) using engine Mascot v.2.3.01 (Matrix Science Ltd.), with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions.

Surface free energy (SFE)

Surface free energy (mJ/m^2) of coated resin specimens (n=8) was determined by means of acid-base technique previously described by Combe *et al*., 2004. Shortly, coated specimens were dried at room temperature and then subjected to SFE evaluation by using the Goniometer Ramé-Hart 500 (Ramé-hart Instrument co., NJ, USA). Sessile droplets (15 µL) of three probe liquids were recorded and automated measured for its contact angle in the point of air-liquid-specimen intersection, in an environment at 25±1℃ and 50±5% relative humidity. The dispersive Lifshitz-van der Waals SFE component (γ_s^{LW}) was determined by the cosine of the contact angle of the non-polar probe liquid 1-bromonaphthalene. In addition, the polar Lewis acid-base (γ_s^{AB}) SFE component was achieved by the contact angle of the formamide and distilled water droplets, both polar liquids (van Oss *et al*., 1988).

Assay of *C. albicans* expression of virulence factors

Cultures of *C. albicans* (ATCC 90028) were reactivated from their original broth by its incubation (24 hours/37℃) in Sabourau d Dextrose Agar (SDA). From the grown colonies, a loop of yeasts was harvested and incubated (18 hours/37ºC/75 rpm) in 25 mL of sterile Yeast Nitrogen Base broth (YNB)

supplemented with 50 mM glucose (Becton Dickinson, Franklin Lakes, NJ). Subsequently, *Candida* cells were washed with PBS by centrifugation (6000 *g*/10 min) and a final suspension of 1 x 10^7 cells/mL was prepared in YNB 100 mM glucose, ascertained spectrophotometrically (absorbance of 0.25 at 520 nm) (Spectronic 20; Bausch & Lomb, Rochester, NY, US).

Biofilm was developed over the WS and WSBP coated resin discs placed vertically in pre-sterilized 24-well tissue culture plate with 2 mL of the standardized inoculum for 72 hours (37ºC/75 rpm). After 1.5 h and at every 24 hours the specimens containing the biofilms were gently washed in PBS and transferred to new plates containing fresh broth. At 1.5, 24, 48 and 72 hours the biofilm of each specimen was disrupted individually by sonication into 3 mL of sterilized PBS (7 watts/30 s). The suspension was then centrifuged and the pellet was resuspended in 100 µL of PBS.

C. albicans hydrolytic enzymatic production was assessed by the inoculation of 5 µL of the suspension on plates of reduced-egg yolk agar for phospholipase (Price *et al*., 1982) or reduced-BSA-Agar for proteinase (Rüchel *et al*., 1982). After incubation (37ºC/48 h) the ratio among the diameter of the colony and the diameter of the colony plus the halo of precipitation formed around it was measured. The lower the ratio the stronger the enzymatic activity (Price *et al*., 1982). Each virulence factor assay was tested in triplicate (n=12), and the experiment was carried out on four separate occasions.

Statistical analysis

Results were statistically analyzed by the SAS/LAB package (SAS Software, version 9.0, SAS Institute Inc., Cary, NC, USA). Error distribution and degree of non-constant variance were tested for ELS and expression of virulence factors. The polar SFE component data were subjected to the square root transformation due to the not normally means distribution. Differences among groups were assessed by one-way ANOVA. For the virulence assays data were subjected to

two-way ANOVA. Tukey HSD was set as the post-hoc test for all variables. Significant level was set at p=0.05.

Results

The spectral analysis resulted in the successful identification of 60 proteins in WS-derived ADP. However, in this study the proteins considered were only those found in at least 2 of 3 experiments being only 27 proteins. In order to achieve the ADP hidropathy of the acquired pellicle, proteins with higher spectral counts for each protein were considered and 77% of the proteome members were hydrophobic (Table 1).

Table 1. Assignment of proteins identification by Q-TOF mass spectrometry from the ADP formed in the presence of whole saliva.

Accession number	Protein assigned	MW (Da)[a]	emPai index[b]			Spectral counts			Hydro philicity[c]	MW
			exp1	*exp2*	*exp3*	*exp1*	*exp2*	*exp3*		
IPI00021439	Actin, cytoplasmic 1	42052	0.08	0.16	0.35	2	2	6	0	5.1
IPI00300786	Alpha-amylase 1	58415	3.9	4.18	0.64	167	165	10	-0.1	6.5
IPI00418512	Glycoprotein 340	170672	0.04	0.04	0.02	2	2	2	-0.1	5.1
IPI00641229	IGHA2 Ig alpha-2 chain C region	37301	0.67	0.98	0.67	11	64	6	-0.1	5.7
IPI00152154	Mucin-7	39432	0.08	0.08	0.08	1	3	4	0.0	9.0
IPI00013890	Isoform 1 of 14-3-3 protein sigma	27871	x	0.12	0.12	x	8	1	0.4	4.5
IPI00027462	Protein S100-A9	13291	x	0.26	1.49	x	1	5	0.4	8.7
IPI00383164	IGHA1 SNC66 protein	54601	0.51	X	0.72	10	x	6	-0.2	6.2
IPI00007047	Protein S100-A8	10885	0.32	X	0.74	1	x	2	0.2	6.6
IPI00025476	1,4 -alpha-D-glucan glucanohydrolase	58354	2.34	2.53	X	123	116	x	-0.1	6.6
IPI00296654	Bactericidal/permeability-increasing p-1	49256	0.07	0.07	X	1	1	x	-0.4	9.0
IPI00003269	Beta-actin-like protein 2	42318	0.08	0.08	X	1	1	x	0.0	5.3
IPI00032294	Cystatin-S	16489	1.11	0.75	X	4	4	x	4.8	0.1
IPI00305477	Cystatin-SN	16579	2.06	0.75	X	7	3	x	0.0	7.0
IPI00178926	Immunoglobulin J chain	18543	0.4	1.73	X	2	6	x	0.2	4.9
IPI00009650	Lipocalin-1	19409	0.9	0.62	X	5	4	x	0.1	5.2
IPI00004310	Ly6/PLAUR domain	37031	0.09	0.09	X	1	1	x	0	7.6
IPI00855918	Mucin-5B	611987	0.01	0.02	x	2	4	x	0.2	6.2
IPI00005721	Neutrophil defensin 1	10536	0.76	0.76	x	2	2	x	-0.2	6.8
IPI00004573	Polymeric immunoglobulin receptor	84429	0.58	0.52	x	17	16	x	0.1	5.4
IPI00022974	Prolactin-inducible protein	16847	1.49	1.08	x	11	10	x	-0.2	8.1
IPI00550731	Putative uncharacterized protein	26503	0.43	0.43	x	5	4	x	-0.2	9.5
IPI00022434	Putative uncharacterized protein ALB	73881	2.37	2.68	x	49	60	x	0.2	6.3
IPI00022463	Serotransferrin	79280	0.04	0.04	x	1	1	x	0.1	6.7
IPI00374315	UPF0762 protein C6orf58	38244	0.64	0.51	x	10	10	x	-0.2	5.7
IPI00166729	Zinc-alpha-2-glycoprotein	34465	0.74	0.45	x	14	10	x	0.0	5.6
IPI00060800	Zymogen granule protein 16 homolog B	22725	0.15	0.15	x	1	2	x	-0.2	7.6

The (x) means that the protein was not identified on the respective experiment. a) Refers to the experimental molecular wage (Da) of the protein. b) Exponentially modified protein abundance index (absolute protein amount/number of sequenced peptides per protein), (Ishihama *et al*., 2005). c)Theoretical hydrofilicity of the sequenced peptide.

In contrast, in WSBP-derived ADP 54 proteins were identified. The 15 unequivocally identified members (predominantly hydrophilic, 94%) included albumin and fibrinogen (Table 2). Proteins identified in only one experiment were in absolute numbers, 33 and 39 for WS and WSBP-derived ADP, respectively. These proteins can be assumed as hypothetical constituents of ADP proteome; however, their presence remains unclear.

Table 2. Assignment of proteins identification by Q-TOF mass spectrometry from the acquired pellicle formed onto PMMA after exposure to saliva and blood plasma.

Accession number	Protein assigned	MW (Da)[a]	emPai index[b]			Spectral counts			Hydro philicity[c]	MW
			exp1	*exp2*	*exp3*	*exp1*	*exp2*	*exp3*		
IPI00478003	Alpha-2-macroglobulin	164614	0.04	0.04	0.15	2	2	10	-0.1	6.0
IPI00154742	Ig lambda-2 chain C regions	25119	0.12	0.13	0.28	1	2	2	0.0	7.1
IPI00021885	Isoform I of Fibrinogen α chain	95656	0.13	0.07	0.14	5	2	6	0.2	5.6
IPI00550731	Putative unch. Protein	26503	0.52	0.43	0.81	6	4	11	-0.2	9.5
IPI00022463	Serotransferrin	79280	0.6	0.18	0.69	22	8	15	0.1	6.7
IPI00300786	Alpha-amylase 1	58415	x	0.32	1.68	x	8	28	-0.1	6.5
IPI00298497	Fibrinogen beta chain	56577	x	0,06	0.06	x	1	1	0.1	8.3
IPI00022434	Putative unch. protein ALB	73881	x	3.99	17.36	x	79	237	0.2	6.3
IPI00745872	Isoform 1 of Serum albumin	71317	2.32	x	18.54	104	x	236	0.2	5.9
IPI00021854	Apolipoprotein A-II	11282	1.02	0.3	x	4	1	x	0.0	5.0
IPI00152154	Mucin-7	39432	0.15	0.08	x	3	1	x	0.0	9.0
IPI00386524	IGHA1 CDNA FLJ25298s	54468	0.77	0.6	x	16	8	x	-0.2	6.2
IPI00431645	HP protein	31647	0.7	0.22	x	10	2	5	-0.1	8.4
IPI00555812	Isoform 1 of Vitamin D	54526	0.11	0.06	x	2	1	x	0.2	5.2
IPI00796467	TF 11 kDa protein	11140	1.05	0.31	x	5	3	x	x	X

The (x) means that the protein was not identified on the respective experiment. a) Refers to the experimental molecular wage (Da) of the protein. b) Exponentially modified protein abundance index (absolute protein amount/number of sequenced peptides per protein), (Ishihama *et al.*, 2005). c)Theoretical hydrofilicity of the sequenced peptide.

Total SFE, Lifshitz-van der Waals (γ_s^{LW}) and the Lewis acid-base (γ_s^{AB}) components of the denture base material (PMMA) after development of experimental ADP in the presence of WS or WSBP solution are summarized in Table 3. The presence of blood plasma proteins in ADP increased the total SFE and the γ_s^{AB} ($p<.001$). No difference was observed between both groups regarding the γ_s^{LW} component ($p>.001$).

Table 3. Total surface free energy (γ^{TOT}), Lifshitz-van der Waals (γ_s^{LW}) and Lewis acid-base components (γ_s^{AB}) of ADP pellicles formed on PMMA resin (mJ/m^2); (Mean±SD, n=8).

Groups	γ^{TOT}	(γ_s^{LW})	(γ_s^{AB})
Without pellicle	40.3 ± 1.0 a	40.0 ± 1.0 a	0.3 ± 0.3 a
WS pellicle	35.7 ± 4.3 b	33.6 ± 1.0 b	2.1 ± 4.1 a
WSBP pellicle	43.6 ± 1.4 c	32.9 ± 1.1 b	10.7 ± 1.5 b

Different lowercase letters represent statistical differences among groups (Tukey test; p<.05).

No significant difference was found in *C. albicans* virulence factors expression throughout time, regardless the enzyme analyzed or the ADP constitution (p>.05) (Table 4). In addition, WSBP group showed statistically higher expression of both virulence factors analyzed in all biofilm stages (p<.001).

Table 4. Expression of putative virulence factors of *C. albicans* in the different stages of biofilm development by means of extracellular enzyme activity[a], (means±SD, n=12).

	ADP	Adhesion	24 h	48 h	72 h
Phospholipase	WS	0.30 ± 0.01 Aa	0.31 ± 0.02 Aa	0.32 ± 0.00 Aa	0.30 ± 0.01 Aa
	WSBP	0.23 ± 0.01 Ab	0.24 ± 0.02 Ab	0.25 ± 0.01 Ab	0.23 ± 0.01 Ab
Proteinase	WS	0.52 ± 0.03 Aa	0.48 ± 0.03 Aa	0.56 ± 0.02 Aa	0.58 ± 0.03 Aa
	WSBP	0.42 ± 0.02 Ab	0.47 ± 0.03 Aa	0.44 ± 0.03 Ab	0.49 ± 0.01 Ab

Uppercase letters represent statistical comparison trough time. Lowercase letters demonstrate statistical differences among WS and WSBP derived-pellicles within each time (Tukey test; p<.05). a. Ratio of colony diameter by the enzymatic-depositing halo plus colony diameter. Therefore, the higher the index value, the smaller the enzymatic activity.

Discussion

The novelty of this study is the exploration regarding the proteome of ADP formed in the presence of human blood plasma, and its role in the substrate properties and *C. albicans* biofilms. Although some proteins as α-amylase, albumin, lysozyme, IgA, IgG and peroxidases have previously been identified in pellicles formed on PMMA substrata (Edgerton *et al*., 1992; Goeck *et al*., 2002), the proteomic ADP fingerprint have not yet been achieved (Edgerton *et al*., 1992; Yoo *et al*., 2003). For example, important antifungal proteins as cystatins, proline-rich proteins (PRPs), and mucins have never been identified as constituents of the acquired pellicle formed on PMMA resin.

The use of state-of-the-art Quadrupole Time-of-flight (Q-TOF) mass spectrometer (LC-MSMS) (Lendenmann *et al*., 2000; Yao *et al*., 2003) allowed the identification of 27 different proteins in the WS-derived ADP proteins. The presence of low molecular weight proteins as MUC7 and MUC5B, protein S100 (A8, A9), neutrophil defensin and immunoglobulins supports the assumption that this pellicle represents an innate defense barrier against *C. albicans* colonization (Dodds *et al*., 2005). In addition, we identified cystatins-S and cystatin-SN, proteins with biological functions related to the suppression of tissue proteolysis in response to inflammatory processes as showed by Dickinson *et al*., 2002.

In contrast, in WSBP-derived ADP a different proteome profile was observed. Albumin and fibrinogen proteins constituted the major differences, not only by their relative higher abundance but also by its functional properties. Fibrinogen is assumed as an important factor involved in the development of candidosis due binding proteins specifically localized on the cell wall surface of *C. albicans* germ tubes (Annaix *et al*., 2006). Either albumin or its fractions are also considered to trigger *C. albicans* virulence (Feng *et al*., 1999). Interestingly, in WS-derived ADP a predominance of hydrophobic proteins was noticed. In contrast, a hydrophilic character was attributed to the WSBP-derived ADP. This new evidence is supported by the SFE results which revealed that the presence of blood plasma proteins significantly enhanced the surface energy and polarity of the surface

substrata. Previous studies identified a strong positive correlation between SFE and adhesion of fungal cells (Minage *et al*., 1985; Hahnel *et al*., 2009). This can be due the binding of phosphate groups on the intraoral hard surfaces (Nikawa *et al*., 2000; Pires *et al*., 2001) and the high hydrophilicity of *C. albicans* cell wall (Luo and Samaranayake, 2002). Whilst, adherence of microorganisms increases proportionally to surface wettability (Minagi *et al*., 1985), other factors such as surface roughness and ADP protein folding may interfere in the adhesion process. Nevertheless, this assumption needs to be critically evaluated in future researches.

Although the relationship between ADP fingerprint profile and yeast cells adherence remains unclear, the binding of blood plasma proteins onto the denture resin enhanced the expression of *C. albicans* virulence factors. Based on the present results, WSBP-derived ADP up regulated both hydrolytic enzymes analyzed, key virulence factors responsible for tissue damage, fungal dissemination and host local immune system suppression (Karkowska-Kuleta *et al*., 2009). For instance, the functional impact of the WSBP proteome in *C. albicans* biofilm virulence may be due the triggering effect of its components on intracellular transcriptional reactions, as shown by (Mukherjee *et al*., 2003).

Another important finding is the maintenance of the ADP virulence inductive effect during all *C. albicans* biofilm stages of development, which in terms may explain the perpetuation and recurrence of *Candida*-associated denture stomatitis despite the conventional therapeutic approaches (Spellber *et al*., 2006). In clinical terms, the findings imply the necessity of resin surface modifications or treatments that promotes a selective protein adsorption, therefore modulating the fungal infection at the acquired pellicle level. Although, the *in vi*tro condition of the present study does not fully mimic the proteolytic environment of the oral cavity, the present study provides relevant data regarding ADP proteome, which can impacts on the pathophysiology of the most common oral disease among denture wears (Barbeau *et al*., 2003).

Conclusion

The presence of blood plasma proteins determined different ADP protein profile which increased SFE of PMMA and *C. albicans* biofilms virulence.

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CAPÍTULO III

Acquired pellicle effects on structure, bioactivity and virulence-factors of *Candida albicans* biofilms

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Abstract

Objective: Since the effect of specific proteins presents on acquired denture pellicle on the, biofilm structure, bioactivity and virulence factors of *Candida albicans* biofilms is unknown, this study was conducted. Design: *C. albicans* biofilms were grown on poly(methylmethacrylate) discs covered with single-protein pellicles formed with albumin, mucin I, mucin II, lactoferrin, fibrinogen, C3b, IgA or histatin 5. Whole saliva pellicle was set as the control group. Methods: Biofilm structure was analyzed by confocal scanning laser microscopy, bioactivity was measured by XTT reduction assay and the activities of *C. albicans* virulence factors, phospholipase, hemolysin, lipase and proteinase were also determined (n=12). All assays were made at 1.5 h (adhesion phase), and at 24, 48, and 72 h of biofilm growth. Biofilm structure and bioactivity data were analyzed by two-way ANOVA and the virulence activity by one-way ANOVA. Tukey's test was use as post ANOVA comparisons, at 5%. Results: The pellicles of mucin I, mucin II and histatin 5 interfered with the biofilm structure, increasing the average diffusion distances and mean thickness of the *C. albicans* biofilms ($P < 0.001$) from the adhesion phase to the 24 hours. At the adhesion phase, all single-protein pellicles presented higher bioactivity than the salivary pellicle ($P < 0.001$). Although at 24 hours of biofilm development there was a significant decrease in the bioactivity in comparison to the adhesion phase, all pellicles leaded to higher bioactivity levels than the control group ($P < 0.001$), excepted for Mucin I. In addition, compared to all other single-protein pellicles, lactoferrin, IgA, and histatin 5 pellicles inhibited the activity of all virulence factors tested. Conclusion: The findings suggest that *C. albicans* biofilm structure, bioactivity and virulence factors may be affected by type of protein present in the pellicle adsorbed on dentures surface.

Keywords: Acquired pellicle; Saliva; Blood plasma; Bioactivity; Confocal microscopy; Virulence; *C. albicans*.

Running title: Acquired pellicle and *C. albicans*

Introduction

Proteins derived from saliva and human blood plasma exhibit a significant antifungal activity against *C. albicans*, which may explain the down regulation of this commensal yeast in the oral cavity of healthy individuals (1, 2). Although the anticandidal activity of saliva-derived proteins has been shown by using planktonic strains, the effect of these proteins after adsorption onto the oral surface has not been reported for *C. albicans* biofilm development and virulence factor activity.

The protein pellicle formed on the resin surface of oral prosthesis, the acquired denture pellicle, contains a large number of proteins that may enable its protective behavior against microbial colonization. For instance, among other salivary glycoproteins identified, amylases, mucin (MG1), lysozyme, albumin, and IgA are able to modulate *C. albicans* colonization on various biological surfaces (3).

Significant data suggests that modifications of pellicle proteins intrinsic to the adsorption process may alter their functional properties (4). In contrast, a recent study has suggested that even during the early proteolysis phase of some proteins, such as histatin 5, antifungal properties are associated with the intact protein (5). Additionally, recent evidence has shown that histatin 1, when adsorbed to the substratum, resists to proteolytic degradation (6). However, these pellicles proteins have not been linked to any mechanisms regarding the control of *C. albicans* biofilm development.

Moreover, few studies have examined the development of *C. albicans* biofilms; and how pellicle's proteins such as albumin, fibrinogen, C3b, and others are related to fungal virulence has not been determined. *Candida* species, usually found in the oral microflora, shift from their commensal relationships to pathogenic actions by disrupting the oral environment balance (7, 8). Besides the polymorphic yeast-hyphal transition, the production of hydrolytic enzymes may be a final consequence of biofilm development in response to an external stimulus, which is known to affect the pathogenicity of *Candida* yeasts (9, 10). However, the effect of

these adsorbed salivary or plasmatic proteins to denture acrylic surfaces have been linked to the hydrolytic activity of *C. albicans* biofilms.

Therefore, the present study aimed to assess the effect of single-proteins acquired pellicles adsorbed to acrylic resin on the metabolic activity and cellular structure of *C. albicans* biofilms. In addition, the impact of this possible modulation on virulence factor activity was assessed using a multi-enzymatic approach.

Materials and Methods

Experimental Design

This *in vitro* study had a randomized and blinded design for the variables of average diffusion distances, mean thickness, bioactivity and virulence factor activity. Poly(methylmethacrylate) (PMMA) resin discs were randomly allocated by lottery to one of following groups of acquired pellicles: salivary (control group), albumin, mucin I, mucin II, lactoferrin, fibrinogen, C3b, IgA, or histatin 5 (experimental groups). A numerical code system was used in order to make the examiners blind for the groups of study during the data acquisition. Pellicle composition (salivary and other single-protein pellicles) and stages of biofilm development (the four time-points) were set as study factors for the biofilm structure analysis, characterized by confocal laser microscopy and the bioactivity of the *C. albicans* biofilms (ATCC 90028). Moreover, four independent experiments in triplicates were made (n=12). For the activity of hydrolytic enzymes (phospholipase, hemolysin, lipase and proteinase) two independent experiments in triplicate were conducted (n=24) and the pellicle composition was consider as the study factor.

Acrylic discs preparation

The heat-cured PMMA resin used in this study was manipulated according to the manufacturer's instructions at room temperature in an environment with approximately 50% relative humidity. Samples with standardized size (10 mm in diameter and 2 mm in thickness) were made using a stainless steel matrix. After

the polymerization cycle (20 min, water bath at 100℃), the matrix was left to cool for 3 h and the resulting PMMA samples were immersed in distilled water at 37℃ for 12 h for residual monomer release (11). In order to avoid bias due to the topography of the samples, their surfaces were polished by a set of aluminum oxide papers (320, 400, and 600-grit) mounted in a horizontal polisher (APL-4; Arotec, Sao Paulo, Brazil). Measured by profilometry (Surfcorder SE1700; Kosaka Laboratory Ltd, Tokyo, Japan), the grinding protocol allowed the standardization of 120 the surface roughness (Ra) to be 0.32 ± 0.03 μm.

After finishing, the samples were coupled to metallic holders, allowing the assays to be performed in the vertical position. Then, they were disinfected in a 0.5% sodium hypochlorite bath (5 min, under agitation) followed by three sets of ultrasonic cleaning with distilled water for 20 min each (Thornton T 740; Thornton-Inpec Eletronica Ltda, Vinhedo, Brazil), which was a protocol adapted from Dandakery *et al*., 2003 (12).

Single-proteins and salivary acquired pellicle formation

Acrylic discs were subjected to pellicle formation prior to each experimental assay. For the development of the salivary acquired pellicle, stimulated whole saliva (induced by mastication of Parafilm M; American Can Co, Neenah, WI, USA) was collected in the same period of the day in ice-chilled polypropylene tubes. After collection, samples from four individuals were mixed, clarified by centrifugation (10.000 *g*/5 min/4℃), filtered through a 0.22 μm membrane filter (Corning, NY, USA), and treated with a protease inhibitors (0.1 mM EDTA and 0.1 mM phenylmethanesulfonyl fluoride) (Sigma Aldrich Co., St. Louis, MO, USA). All the donors gave formal consent in accordance with the protocol (042/2008) previously approved by the Research and Ethics Committee of Piracicaba Dental School, State University of Campinas.

The single-protein pellicles were formed after exposure of the acrylic discs to a solution of the protein of interest in a saline solution (2 mM CaCl2, 3 mM NaH2PO4, and 0.2 mM NaHCO3; pH 7.4) prepared according to Birkeland *et al*.

(1973)(13), with minor modifications. The investigated proteins (albumin a9647, mucin-Type I m3895, mucin-Type II m2378, lactoferrin l4765, fibrinogen f3879, complement C3b c2910, Iga i1010 and histatin 5 h6027) were reconstituted according to the manufacturer's instructions (Sigma Aldrich Co., St. Louis, MO, USA). Final protein concentrations were established based on published data in order to mimic their availability in saliva (Table 1).

Table 1. Protein concentration (mg/L) in the solutions used to form acquired pellicle and the respective references.

Proteins	Concentration	Reference
Albumin	40	Bürgers R. *et al.*; 2010 (14)
Mucin I	233	Rayment S. A. *et al.*; 2000 (15)
Mucin II	133	Rayment S. A. *et al.*; 2000 (15)
Lactoferrin	5	Dipaola C. *et al.*; 1980 (16)
Fibrinogen	80	Scheidelera L. *et al.*; 2007 (17)
C3b	30	Potempa M. *et al.*; 2009 (18)
IgA	156	Strazdins L. *et al.*; 2005 (19)
Histatin 5	308	Sugimoto J. *et al.*; 2006 (20)

Sterilized acrylic discs were placed vertically into a well of a pre-sterilized flat bottomed 24-well tissue culture plate and incubated with 2 mL of each proteic solution for 2 h at 35 ± 2℃, in order to mimic the oral environment. To remove any unbound protein, after the incubation period, each sample was gently washed twice in a new well containing 2 mL of sterilized purified water for 2 sec and then transferred to a new plate for the subsequent assay.

Inoculum and biofilm development

Prior to the experimental assays, *C. albicans* (ATCC 90028) was reactivated *a*erobically on sabouraud dextrose agar (37℃, 24 h) and then transferred to yeast nitrogen based broth (YNB) (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose. After incubation (37℃/24 h/75 rpm), the growing cells were washed twice with 25 mL of phosphate buffered saline (PBS), pH 7.4 (20℃/4

min/6000 *g*). In order to standardize the inoculum, the washed pellet was resuspended in YNB supplemented with 100 mM glucose and the cell concentration was optically adjusted (UV spectrophotometer; Beckman Coulter Inc., Fullerton,CA, USA) to an absorbance of 0.25 at 520 nm (10^7 cells/mL).

The biofilms were developed on protein-coated PMMA samples placed vertically in a 24-well polystyrene cell culture plate (TPP, MIDSCI, St. Louis, MO, USA) containing 2 mL of the standardized inoculum solution. The biofilms were allowed to develop (incubation at 37℃, 75 rpm) fro m the adhesion phase until 72 h of maturation. Four sets of independent experiments (considering the time of biofilm development) were conducted at the same time in order to perform the assays under the same experimental conditions (inoculum concentration, incubation sets, and wash procedures). After 1.5 h and every 24 h thereafter, after the cultivation began, all the biofilms were washed twice by dipping the samples in a wheel with sterile PBS. Immediately, they were placed in a new plate containing 2 mL of fresh YNB broth supplemented with 100 mM glucose and re-incubated under the same conditions.

Biofilm structural analysis

After each time-point of *C. albicans* biofilm development, acrylic discs with their respective grown communities were incubated with the nucleic acid stains SYTO-9 and propidium iodide (Live/Dead *BacLight* viability kit; Invitrogen-Molecular Probes, Eugene, OR, USA). The plates were incubated at 30 ℃ for 20 min and protected from light by covering the plates with an aluminum foil. Biofilm structural analysis was carried out using confocal laser scanning microscopy (CLSM) (Leica Microsystems CMS, Mannheim, GE). Five randomly selected spots on each sample (n = 12) were scanned (at 1 μm intervals 180 in the z-axis). The resulting series of images was subjected to COMSTAT software analysis, which allows the quantification of average diffusion distances and mean thickness of the biofilm (21).

Bioactivity Assay

At each time-point, the resin discs (n=12) with the biofilms were transferred to a new 24-well polystyrene cell culture plate containing 2 mL/well of a solution of 1 mM XTT (C22H16N7NaO13S2; MW 673.52) (9). Briefly, a base solution of 1 mM XTT (Sigma Aldrich Co., St. Louis, MO, USA) was prepared by mixing the salt with sterile PBS supplemented with 200 mM glucose. The XTT solution was filtered through a 0.22 µm membrane (Corning, NY, USA) and stored at -70℃ until use. Prior to the assay, the XTT solution was mixed with 0.4 mM menadione (Sigma Aldrich Co., St. Louis, MO, USA) at a ratio of 20:1 (v:v). Afterwards, the biofilms were incubated for 3 h and protected from light exposure (37℃/75 rpm). After the incubation time, each solution was centrifuged and the supernatants were transferred to polypropylene disposable spectrophotometer cuvettes. The colorimetric changes, as a result of the conversion of the tetrazolium dye into a colored formazan derivative, were measured at 490 nm using a spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

Determination of Candidal hydrolytic enzymatic activity

Prior to the assay of virulence, at each time point (1.5, 24, 48 and 72 h) the biofilms on the resin discs were disrupted by sonication at 7 W for 30 sec into 1 mL of sterilized PBS (22). The resulting fungal cell suspension was then centrifuged (3.000 rpm/1 min/room temperature) and washed twice with PBS. The remaining pellet was resuspended in 100 µL of PBS. Yeast suspension droplets (5 µL; with approximately 108 203 cells/mL) were dispensed in triplicate onto specific medium in order to evaluate enzyme production. In brief, the extracellular phospholipase activity was determined on egg yolk agar (23); the hemolysin assay was based on the digestion of an agar medium supplemented with fresh sheep blood (24); lipase activity was assessed by the tween opacity test (25); and the proteinase activity was evaluated by means of BSA degradation according to Rüchel *et al*. (1982)(26).

The plate cultures were all incubated aerobically at 209 37 ± 2ºC for the following times: 48 h (phospholipase); 20 days (proteinase); 7 days (hemolysin);

and 7 days (lipase). The enzymatic activity of phospholipase, hemolysin, lipase and proteinase were obtained by dividing the colony diameter by the sum of the colony diameter and the halo of enzymatic precipitation. This ratio, expressed as the enzymatic index, represents the intensity of enzyme production by *C. albicans*. Therefore, a ratio equal to 1.0 indicates the absence of enzymatic activity.

Statistical analysis

The structural and bioactivity variables were statistically analyzed using SAS software v.9.0 (SAS Institute Inc., Cary, NC, USA). The assumptions of equality of variances and normal distribution of errors were checked and the data were transformed as suggested by the software: diffusion distances (log10); mean thickness (square root); bioactivity (exponentiation,$y^{2.3}$); phospolipase (exponentiation, $y^{-0.5}$); hemolysin and proteinase (log10) and lipase (1/y). Biofilm structural variables and bioactivity were assessed by two-way ANOVA, considering pellicle type and biofilm phase as factors. Post hoc comparisons were made using Tukey's test. Enzymatic activities were subjected to one-way ANOVA and the pairwise comparisons by Tukey's test. All statistical analyses were conducted at a 5% significance level.

Results

The biofilm structure data are displayed in Table 2, and significant differences ($P < 0.05$) were detected between adhesion and 24 hours for all pellicles. Mucin I, mucin II and histatin 5 leaded to an increasing in average diffusion distances and mean thickness ($P < 0.05$) from adhesion to 24 hours. No significant differences were observed among the pellicles compared to salivary pellicle (control group) ($P>0.05$) at adhesion phase. In contrast, at 24 hours fibrinogen and histatin 5 pellicles showed higher biofilm diffusion distance and mean thickness compared to the salivary. pellicle (*P<0.05*). Representative images of tridimensional reconstruction of *C. albicans* biofilms developed on fibrinogen and histatin 5 at adhesion and 24 hours are shown in Fig. 1.

Table 2. Diffusion distances (µm) and mean thickness (µm) of *C. albicans* biofilms according to acquired pellicle and time of biofilm growth; (mean ± SD;n=12).

Type of Pellicle	Diffusion distances		Mean Thickness	
	1.5 h	24 h	1.5 h	24 h
Salivary	7.7 ± 0.7 A ab	8.6 ± 2.6 Aac	13.8 ± 1.6 A ab	15.3 ± 5.3 Aac
Albumin	9.1 ± 0.9 A a	10.2 ± 2.4 Aabc	16.2 ± 1.8 A a	18.1 ± 4.3 Aabc
Mucin I	7.7 ± 1.0 A ab	10.3 ± 1.3 Bab	13.5 ± 2.1 A ab	18.7 ± 2.7 Bab
Mucin II	7.9 ± 1.6 A ab	10.4 ± 1.0 Bab	13.9 ± 3.2 A ab	18.8 ± 2.0 Bab
Lactoferrin	7.2 ± 0.7 A ab	9.5 ± 1.6 Aabc	12.4 ± 1.4 A ab	15.9 ± 1.9 Aabc
Fibrinogen	9.4 ± 0.7 A a	11.3 ± 1.7 Ab	16.8 ± 1.4 A a	20.7 ± 3.5 Ab
C3b	7.5 ± 1.8 A ab	7.5 ± 1.3 Ac	13.1 ± 3.5 A ab	13.1 ± 2.6 Ac
IgA	7.4 ± 0.9 A ab	7.7 ± 1.6 Ac	12.9 ± 1.8 A ab	13.4 ± 3.5 Ac
Histatin 5	6.6 ± 0.5 A b	11.6 ± 1.6 Bb	11.3 ± 1.1 A b	21.3 ± 3.2 Bb

Upper case letters represent statistically significant differences among the four time-points. Different lower case letters show statistical significances among the pellicles (Tukey test; $P < 0.05$).

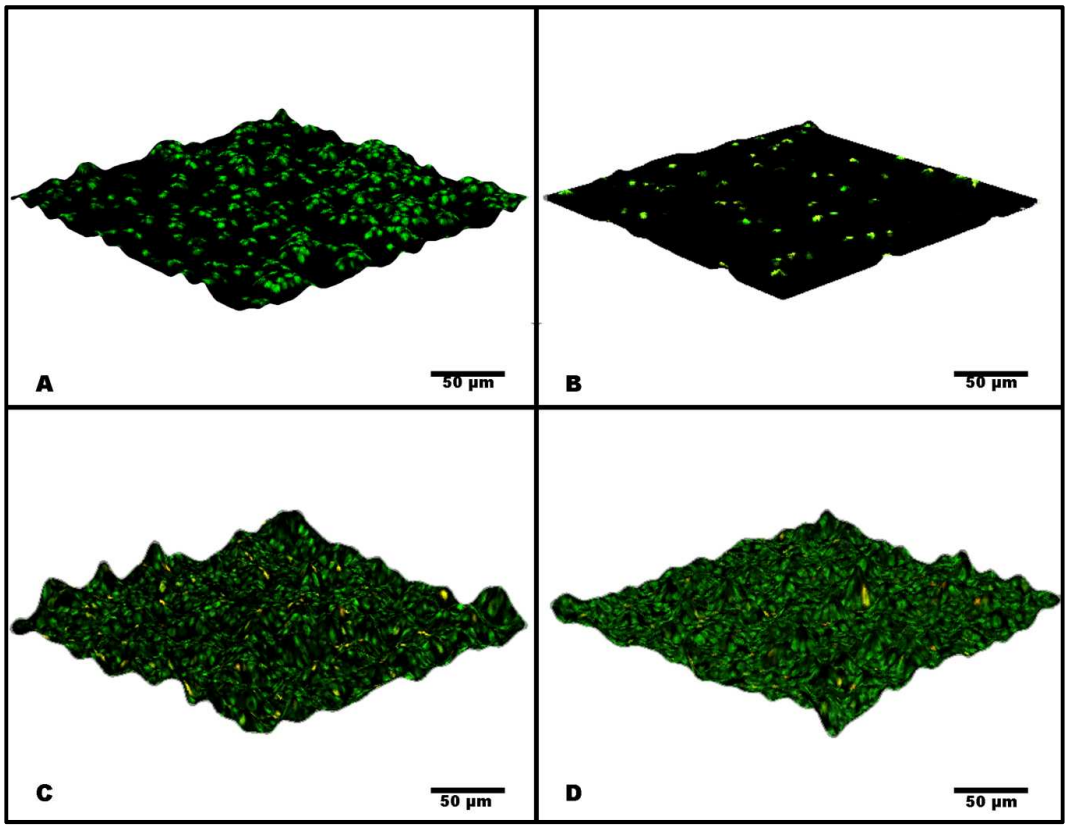

Figure 1. CLSM 3D reconstruction of z-slices showing the topology of biofilms of *C. albicans* biofilms grown during 1.5 and 24 h over acquired pellicles of fibrinogen (A-1.5 h; C-24 h) and histatin 5 (B-1.5 h; D-24 h) formed on acrylic resin. Live cells appear green while distance between cell groupings is black.

The bioactivity results are shown in Table 3. At the adhesion 238 phase, all the pellicles stimulated the bioactivity of *C. albicans* biofilms when compared to salivary pellicle. After 24 hours of biofilm development, all the pellicles except Mucin I induced a reduction in bioactivity compared to the control group ($p<0.05$). However, for 48 and 72 hours there was not a homogeneous behavior of the pellicles, this one is distinct among pellicles, while some presented an increasing metabolic activity at 48h and decreasing at 72 hours, others showed an inverse behavior.

Table 3. Mitochondrial oxidative activity (absorbance values) of *C. albicans* biofilms according to the type of acquired pellicle and time of biofilm growth; (Mean ± SD; n=12).

Type of Pellicle	**Time (h)**			
	1.5	**24**	**48**	**72**
Salivary	0.13 ± 0.01 Aa	0.05 ± 0.00 Ba	0.02 ± 0.00 Ca	0.17 ± 0.00 Da
Albumin	0.87 ± 0.01 Ab	0.28 ± 0.01 Bb	0.05 ± 0.01 Cb	0.12 ± 0.01 Db
Mucin I	0.82 ± 0.01 Ac	0.05 ± 0.00 Ba	0.19 ± 0.01 Cc	0.27 ± 0.01 Dc
Mucin II	1.65 ± 0.01 Ad	0.20 ± 0.00 Bc	0.09 ± 0.00 Cd	0.22 ± 0.00 Dd
Lactoferrin	0.14 ± 0.00 Ae	0.01 ± 0.00 Bd	0.04 ± 0.01 Cb	0.13± 0.01 Db
Fibrinogen	0.26 ± 0.00 Af	0.12 ± 0.01 Be	0.05 ± 0.00 Cb	0.22 ± 0.00 Dd
C3b	0.50 ± 0.00 Ag	0.11 ± 0.01 Be	0.13 ± 0.00 Ce	0.05 ± 0.00 De
IgA	0.92 ± 0.00 Ah	0.08 ± 0.01 Bf	0.17 ± 0.01 Cf	0.21 ± 0.00 Dd
Histatin 5	0.55 ± 0.00 Ai	0.10 ± 0.01 Be	0.12 ± 0.00 Ce	0.07 ± 0.01 Df

Different upper case letters indicate statistical differences among time-points. Lower case letters show significant differences among the types of acquired pellicles ($P < 0.001$). ANOVA Two-way, Tukey test.

Enzymatic activity was not affected by the time factor of biofilm formation ($p>0.05$). However, the type of pellicle interfered on enzymatic activity ($P < 0.001$) (Table 4). Lactoferrin, IgA and histatin 5 pellicles significantly decreased the activity of all enzymes studied ($P <0.05$). In contrast, fibrinogen and C3b pellicles determined the highest levels of *C. albicans* virulence activity ($P <0.05$). Also, it

was observed that salivary pellicle, albumin, mucin I and mucin II pellicles leaded to similar rates of *C. albicans* virulence activities.

Table 4. Enzyme activity* of *C. albicans* biofilms grown on acrylic resin according to the type of acquired pellicle (means±SD; n=24).

Type of pellicle	Enzyme			
	Phospholipase	Hemolysin	Lipase	Aspartyl-protease
Saliva	0.31 ± 0.01 A	0.43 ± 0.07 A	0.52 ± 0.04 AB	0.53 ± 0.05 AC
Albumin	0.33 ± 0.02 B	0.46 ± 0.07 A	0.52 ± 0.06 AB	0.58 ± 0.04 B
Mucin I	0.33 ± 0.01 B	0.44 ± 0.07 A	0.51 ± 0.08 B	0.55 ± 0.04 A
Mucin II	0.31 ± 0.02 A	0.42 ± 0.07 A	0.53 ± 0.06 A	0.51 ± 0.05 C
Lactoferin	0.65 ± 0.04 C	0.72 ± 0.09 B	0.75 ± 0.13 C	0.65 ± 0.03 D
Fribrinogen	0.24 ± 0.03 D	0.36 ± 0.07 C	0.44 ± 0.08 D	0.48 ± 0.06 E
C3b	0.23 ± 0.02 D	0.35 ± 0.07 C	0.49 ± 0.09 E	0.47 ± 0.05 E
IgA	0.68 ± 0.07 C	0.67 ± 0.07 BD	0.73 ± 0.12 C	0.74 ± 0.05 F
Histatin 5	0.70 ± 0.06 C	0.63 ± 0.07 D	0.77 ± 0.11 C	0.68 ± 0.04 D

Diferent letters show significant differences among the types of acquired pellicles ($P < 0.001$).

* Ratio of colony diameter to the total diameter of the colony and halo of precipitation.

Discussion

The pathogenesis of *Candida*-associated denture stomatitis is not well known. In the present study, It was evaluated the role of single-proteins acquired pellicles adsorbed to acrylic resin on the *C. albicans* biofilm structure, and the impact of this possible modulation on virulence factor activity was assessed using a multi-enzymatic approach. The *in vivo* condition was mimicked by using salivary pellicle as control and single pellicle proteins contained in saliva as experimental pellicles. Our findings demonstrated that the adsorbed single-proteins pellicles exerted control over biofilm structural organization, metabolism activity, and virulence of *C. albicans* biofilm, suggesting that adhesion and subsequent yeast infection could be managed.

So far, growing efforts have been made to characterize the proteome profile of the acquired pellicles formed onto intra-oral surfaces (3). However, the effect of

these proteins on *C. albicans* biofilms has been poorly investigated. For instance, the main consensus is that salivary pellicle and its contents can play a dual role in *C. albicans* colonization (27). In the present study, the quantification of biofilm diffusion distance and mean thickness were made in order to verify the influence of the pellicle constitution on the biofilm structure. The present findings are in accordance with a previous report, which demonstrated that effect of the acquired pellicle is restricted to the early stages of biofilm 268 development and do not determine any structural changes during the following maturational stages (28). Moreover, the structural changes between 1.5 to 24 hours were observed only for the adsorbed mucin I, mucin II, and histatin 5 pellicles, which determined compact, thicker and voluminous biofilms. These results possibly indicates that pellicle effect do not alter the structural conformation of mature biofilms, which follows its natural developmental course after the interaction with the pellicle.

However, although at the adhesion phase all single-proteins pellicles do not change the structure of the biofilms in comparison to the salivary pellicle, histatin 5 pellicle determined biofilms with small diffusion distances and mean thickness in comparison to the albumin and fibrinogen pellicles. Therefore, we can hypothesize that the presence of histatin 5 molecules in the acquired pellicle (5), exerts its direct candidacidal activity on the grown biofilms which interferes with biofilm structural organization on the denture surface.

Regarding the acquired pellicle effects on the bioactivity, our findings demonstrated that compared to the salivary, single-protein pellicles increased the bioactivity for *C. albicans* biofilms at the adhesion phase. This finding may be explained by a higher rate of cell-pellicle interactions and intracellular reactions related to the initial steps of biofilm development. As an example, the presence of a conditioning pellicle has been shown to be related to a higher metabolic activity of *C. albicans* biofilms on the initial stages of development, diminishing after 24 h of biofilm maturation (29, 30). Regarding intracellular mechanisms, the yeast-hyphae dimorphic transition determines *the* chemical stimulation of transcriptional reaction chains. These reactions can result in increased expression of adhesins, such as

HWP1; therefore, cell metabolism is especially increased in the early phase of biofilm development (31)*. Moreover,* C3b and histatin 5 pellicles resulted in biofilms with higher metabolic activities in the adhesion stage. This may be due to the high rate of energy required during the killing cascade chain of *C. albicans* by these proteins, as has been shown for histatin 5 (1).

Interestingly, single-protein pellicles affected differently the bioactivity of *C. albicans* biofilms. Therefore, we can assume that the impact of acquired denture pellicle is protein specific and depends on the developmental stage. It can be partially explained by the dual function that some proteins exert. For example, although IgA and mucins have already been associated to *C. albicans* cell clearance (32, 33); they may act as mediators of *C. albicans* adherence by triggering expression of adhesins in the yeast cell 298 wall. In addition, albumin, mucin II, and fibrinogen are considered to be protein carriers (34); therefore, they can form complexes with other small proteins and then exert a different biological function. However, future research is necessary to confirm such a statement.

In an effort to provide evidence for the characterization of possible anticandidal protein pellicles against *Candida*-associated denture stomatitis, we evaluated *C. albicans* virulence by determining the activity of hydrolytic enzymes. Virulence factors, such as phospholipases and proteinases productions, have been directly related to tissue damage, iron acquisition, and overcoming of the host immune system (35). However, none have been linked to any pellicle's proteins. We demonstrate, for the first time, that *C. albicans* biofilms developed over fibrinogen and C3b pellicles presented high hydrolytic activities for all the enzymes. Previous studies have demonstrated a correlation between enzyme production and cellular adherence (36, 37). Therefore, the increased activity exerted by the fibrinogen pellicle may be related to the activation of receptors on the yeast cell wall, as the site mp58 which increases the stability of the microbe-host interaction (38). For C3b pellicle, its low inhibitory effect on the virulence activity of *C. albicans* biofilms may be explained by cellular mechanisms that inactivate or directly inhibit host complement attack. As demonstrated by Luo *et al*., 2010 (39), the secretion of

the glycosylated protein pH-regulated Ag-1 (*Pra1*) by the yeast cell, a potent complement inhibitor, blocks the C3b proteins onto the *C. albicans* cell wall favoring the immune escape and potentially the biofilm virulence activity. In contrast, lactoferrin, IgA, and histatin 5 pellicles were able to diminish the activity of all hydrolytic enzymes analyzed. This relevant finding suggests that anti-candidal proteins adsorbed onto the surface of oral prosthesis are able to inhibit the virulence of *Candida albicans* biofilms, which in turn can be one of the physiological methods by which saliva controls the yeast infections.

The present findings can contribute for the development of therapeutic approaches such as the development of surface treatments which could promote the selective adsorption of proteins. Regardless of the limitations implicit to this *in vitro* study (due to the lack of reproduction of the proteolytic oral environment), based on the virulence observations, potential proteins could be used to inhibit the expression of *C. albicans* virulence factors, which ultimately determine *Candida*-associated denture stomatitis.

Conclusion

The findings suggest that the structure, bioactivity and activity of the virulence factors of *C. albicans* biofilms grown on the denture acrylic surface may change according to the protein composition of the acquire pellicle formed.

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Conflict of interest statement

All authors declare no conflict of interest.

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CONSIDERAÇÕES GERAIS

A análise funcional da película adquirida formada sobre as superfícies intra-orais é de grande relevância para o preenchimento de lacunas científicas relacionadas aos mecanismos patofisiológicas de doenças biofilmes-dependentes. Existe um interesse crescente nos fatores moduladores que governam as interações entre a célula microbiana e o substrato. No entanto, poucos estudos avaliaram esses fatores, como os constituintes protéicos da película adsorvida, relacionando-os ao desenvolvimento e expressão de fatores de virulência de biofilmes.

Com o advento de metodologias empregadas na análise proteômica, tornou-se possível e confiável a caracterização do perfil protéico de películas adquiridas (Siqueira *et al*., 2009), viabilizando a identificação de proteínas, bem como sua abundância na amostra avaliada, pontos determinantes para o início do entendimento deste integumento acelular (Siqueira *et al*., 2007; Paes Leme *et al*., 2011). Além do caráter composicional, avanços na área nano tecnológica, como o emprego de microscopia de força atômica, provêem evidências relativas à análise de propriedades funcionais e mecânicas de moléculas protéicas, bem como viabilizam a investigação destas durante interações com outras proteínas ou mesmo um substrato (Eibl, *et al*., 2005; Simon *et al*., 2006). Esta abordagem a níveis nanométricos viabiliza, por exemplo, a análise do enovelamento de receptores protéicos presentes na parede celular de células fúngicas (Heinisch *et al*., 2010). Desta maneira, a abordagem multi-metodológica para análise protéica é essencial para a avaliação de sua influência em biofilmes, uma vez que suas propriedades composicionais e estruturais podem determinar mudanças na formação e composição de biofilmes e, conseqüentemente, em patologias como a candidose associada ao uso de próteses (Edgerton *et al*., 1992; Lendenmann *et al*., 2000).

Ainda, é importante salientar que esta camada de compostos orgânicos é formada a partir da adsorção seletiva de componentes presentes no ambiente oral, refletindo em um processo dinâmico e reversível (Egerton *et al*., 1992). Neste

contexto, durante os quadros de candidose associada ao uso de próteses, o caráter inflamatório e exsudativo do processo infeccioso (Lynch *et al*., 1994), podem levar a uma maior incorporação de proteínas de origem plasmática e, conseqüentemente modular sua função biológica, hipótese esta, sustentada por evidências geradas no presente estudo como, a influência do proteoma das películas na energia livre de superfície do substrato, propriedade diretamente associada ao favorecimento da adesão de *Candida* sobre o material (Quirynen e Bollen, 1995; Sipahi *et al*., 2001).

Estudos prévios avaliando o efeito de películas salivares e seus componentes na adesão de *C. albicans*, primeiro passo para o desenvolvimento de biofilmes são contraditórios. Diversos trabalhos reportam que a película salivar adsorvida na resina de PMMA reduz a adesão de *C. albicans* (Bosch *et al*., 2003; Moura *et al*., 2006; Pereira-Cenci *et al*., 2007). Em contra partida, outros achados denotam o aumento nas taxas de colonização em função deste integumento protéico (Edgerton *et al*., 1993; Millsap *et al*., 1999). Esta não concordância sobre a função biológica da película adquirida pode estar diretamente relacionada à variação protéica entre as películas empregadas.

Embora com menor impacto sobre a estruturação do biofilme, pode ser observado que diferentes películas mono-protéicas foram responsáveis por diferentes taxas de bioatividade, achados que corroboram com evidências prévias que apontam para diferenças no metabolismo celular de *Candida*, mas não arquitetura do biofilme (San Millan *et al*., 2000). Ainda, embora não como integumento, um estudo recente demonstrou uma relação direta entre a atividade metabólica e histatina 5, sendo que esta proteína foi responsável por diminuir em 50% a bioatividade do biofilme já desenvolvido (Konopka *et al*., 2010). Esta observação hipotetiza uma possível especificidade funcional exercida pelos diferentes constituintes das películas adsorvidas. Embora sejam raros trabalhos apontando a influência isolada de proteínas de origem salivares e séricas adsorvidas ao substrato de poli(metilmetacrilato) no desenvolvimento de biofilmes de *Candida albicans* (Chaffin *et al*., 1998), esta hipótese pode em parte ser

sustentada por achados prévios que concluem que cada proteína desempenha uma função específica na película adquirida formado sobre superfícies dentais (Hannig *et al*., 2006; Siqueira *et al*., 2007; Vitorino *et al*., 2007; Hara *et al*., 2010)

Ainda, assim como na modulação da atividade metabólica, diferentes integumentos protéicos influenciaram diferentemente a expressão dos fatores de virulência dos biofilmes de *C. albicans* desenvolvidos sobre as mesmas. Embora proteínas do sistema imune do hospedeiro, como imunoglobulinas salivares e séricas não tenham demonstrado eficácia na inibição da atividade de aspartil-proteinase (Naglick *et al*., 2005), os achados do presente estudo demonstram que determinadas proteínas da película adquirida, incluindo IgA, modulam negativamente a expressão dos fatores de virulência de C. albicans. Ainda, esta evidência é reforçada pela corroboração com os achados de bioatividade (XTT), uma vez que este indicador da atividade mitocondrial celular relaciona-se diretamente com a expressão da virulência dos biofilmes de *Candida* e esta, por sua vez, na persistência da infecção fúngica (Hasan *et al*., 2009). Clinicamente, a identificação de receptores peptídicos da película adquirida relacionados aos fatores de expressão de virulência da célula de *Candida* poderá indicar condições de risco para a candidose associada ao uso de próteses.

Frente todas as evidências estabelecidas neste conjunto de trabalhos, podemos conceber que a caracterização do proteoma da película adsorvida à resina de PMMA e sua influência no desenvolvimento de biofilmes de *C. albicans*, tem um significado muito maior do que somente a relevância à patofisiologia da candidose associada ao uso de próteses. A especificidade funcional demonstrada pela mesma frente a variações no ambiente oral delineia possibilidades terapêuticas como o desenvolvimento de superfícies protéticas bioativas, por exemplo, capazes de incorporar seletivamente proteínas como histatina 5, cujo potencial antifúngico é capaz de modular negativamente o desenvolvimento de biofilmes fúngicos. Assim, as evidências geradas no presente estudo contribuem para o estabelecimento de medidas preventivas ou mesmo terapêuticas, a fim de evitar o desenvolvimento e progressão desta condição patológica que em

pacientes imunossuprimidos e/ou idosos frágeis, pode levar a candidemia que está associada à alta taxa de mortalidade (30-40%), aumento do tempo de permanência hospitalar e seus custos associados. (Wey *et al*., 1988; Leleu *et al*., 2002; Cheng *et al*., 2005; Pfaller e Diekema, 2007).

CONCLUSÃO GERAL

De acordo com os resultados obtidos, podemos concluir que a ligação de proteínas plasmáticas à película adquirida determina variações nas propriedades funcionais e mecânicas da mesma, capazes de promover o desenvolvimento de infecções fúngicas. Ainda, cabe salientar que as proteínas adsorvidas às superfícies intra-orais mantêm sua função biológica mesmo após a adsorção ao substrato. Baseado nas evidências geradas por estes trabalhos, pode-se concluir que proteínas salivares e plasmáticas adsorvidas como integumentos protéicos exercem uma função modulatória no desenvolvimento de biofilmes de *C. albicans,* bem como na expressão de fatores de virulência.

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*De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of Medical Journal Editors – Grupo de Vancouver. Abreviaturas dos periódicos em conformidade com o Medline.

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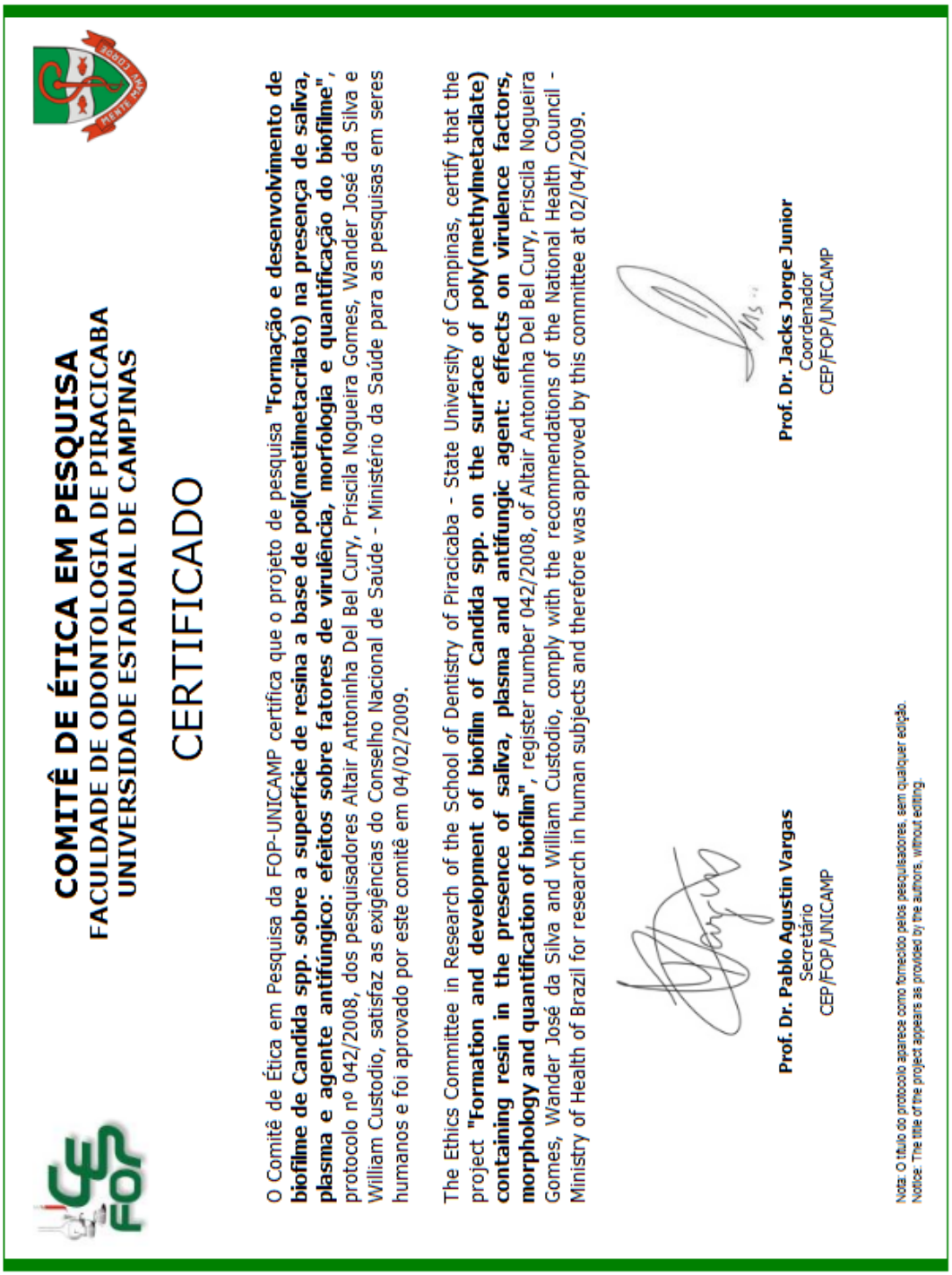
ANEXOS

I - Certificado de Aprovação do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba



COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Formação e desenvolvimento de biofilme de Candida spp. sobre a superfície de resina a base de poli(metilmetacrilato) na presença de saliva, plasma e agente antifúngico: efeitos sobre fatores de virulência, morfologia e quantificação do biofilme"**, protocolo nº 042/2008, dos pesquisadores Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva e William Custodio, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 04/02/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Formation and development of biofilm of Candida spp. on the surface of poly(methylmetacilate) containing resin in the presence of saliva, plasma and antifungic agent: effects on virulence factors, morphology and quantification of biofilm"**, register number 042/2008, of Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva and William Custodio, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 02/04/2009.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

II-Figuras

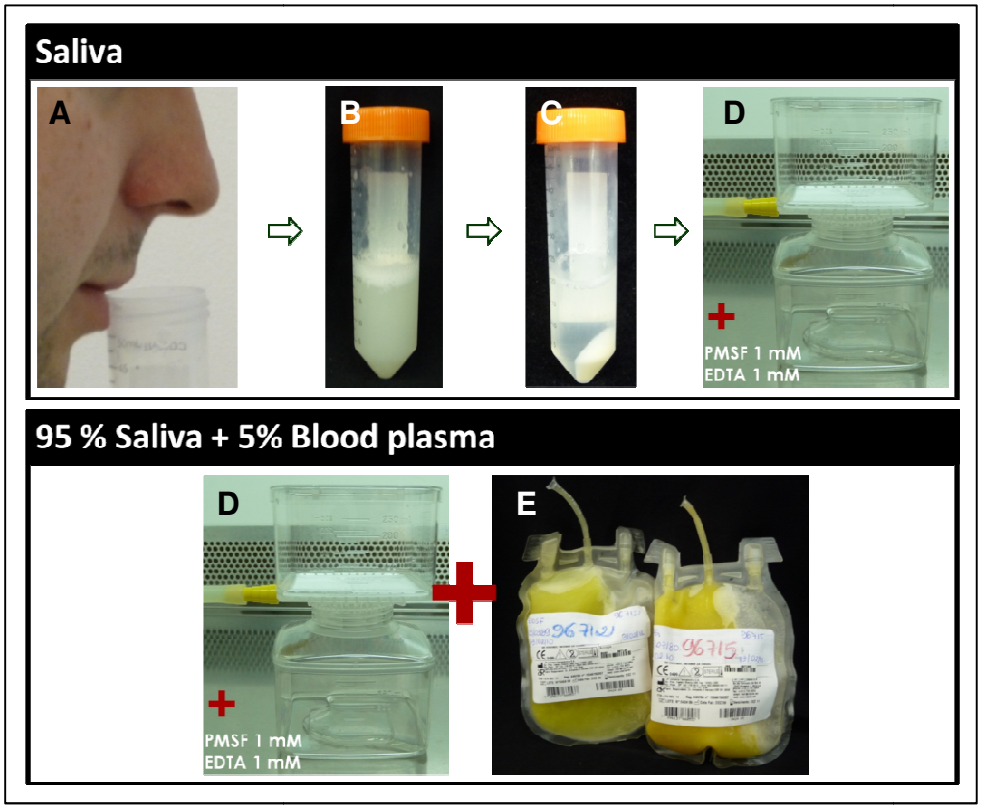


Figura 1. Preparo das soluções experimentais empregadas no Capítulo 1. Coleta de saliva total estimulada (A). Saliva não clarificada (B). Saliva após clarificação (C). Esterilização da saliva por meio de filtragem por membrana 0.22 µm (D). Bolsas de plasma (E).

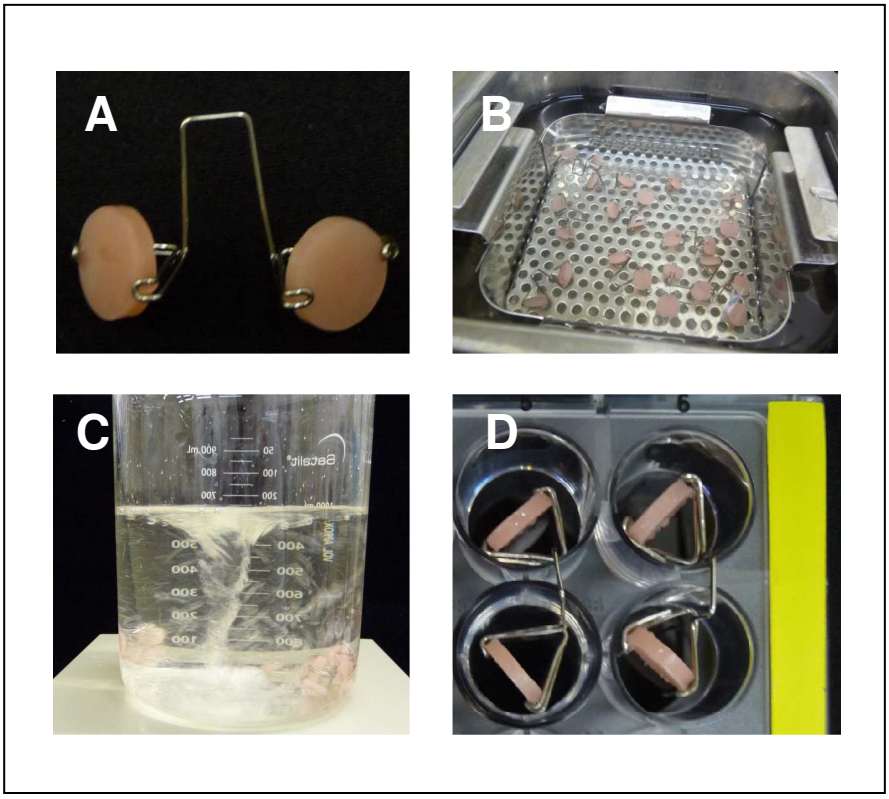


Figura 2. Desinfecção de discos de resina à base de PMMA. Posicionamento dos discos de resina em holders metálicos (A). Banho ultrassonico em água purificada (B). Banho em solução de hipoclorito de sódio 5% (C). Posicionamento vertical dos espécimes alocados em placas de 24 poços para realização dos testes experimentais (D).

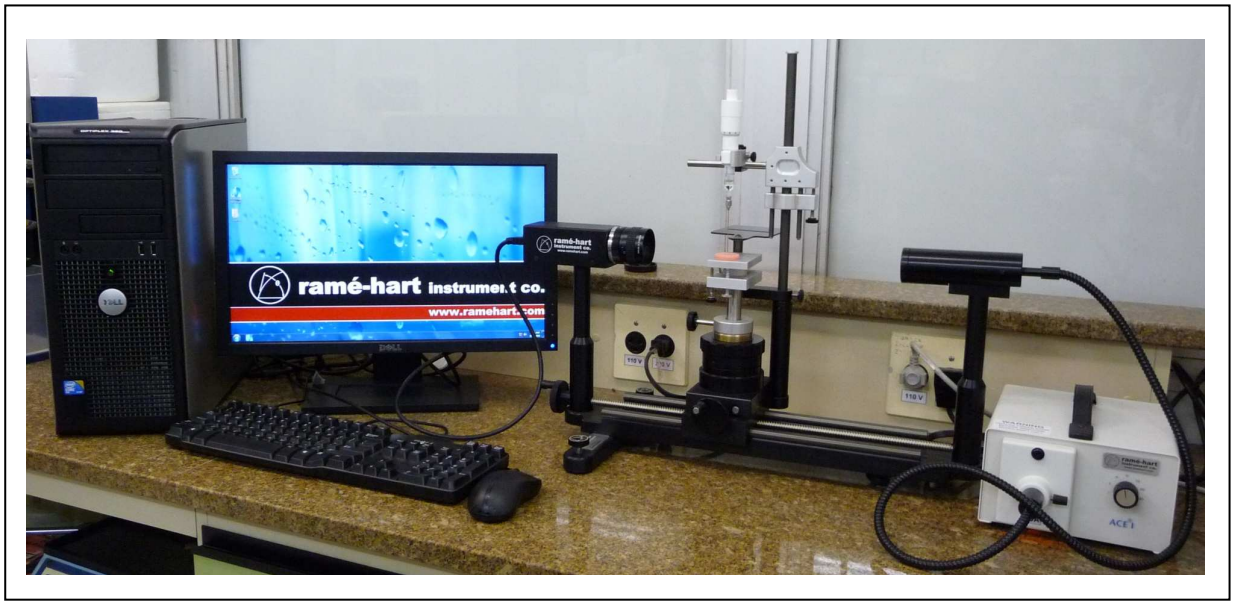


Figura 3. Goniômetro Ramé-Hart 500 (Ramé-hart Instrument co., NJ, USA).

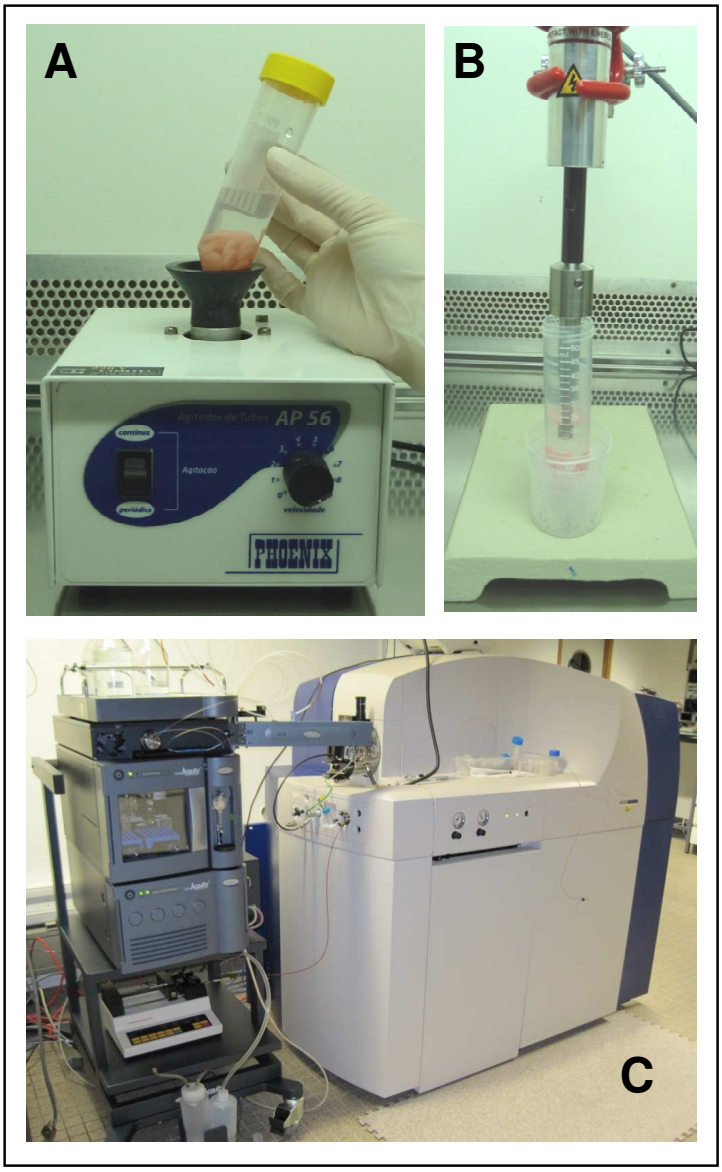


Figura 4. Identificação de proteínas. Vortexação dos discos de resina em água purificada (A). Sonicação de discos de resina imersos em água purificada, em banho de gelo (B). HPLC acoplado ao spectrometro de massas Q-TOF (C).

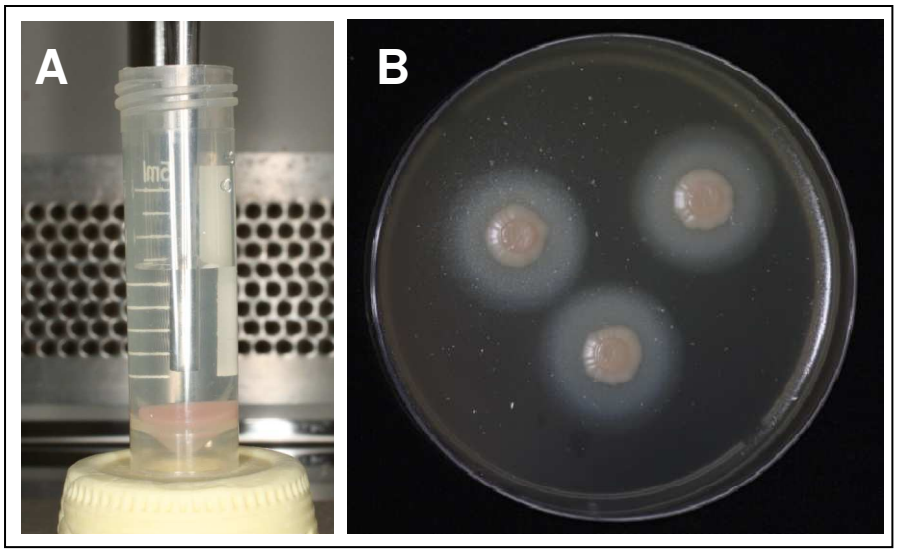


Figura 5. Teste de virulência. Sonicação do espécime em água purificada (A). Exemplo de resposta positiva à produção de fosfolipase (B).

III – Comprovante de submissão do artigo

J. Cell Commun. Signal.
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COMMENTARY

Salivary proteins as predictors and controls for oral health

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Abstract We will provide a translational view of using the recent technological advances in dental research for predicting, monitoring, and preventing the development of oral diseases by investigating the diagnostic and therapeutic role of salivary proteins. New analytical state-of-the-art technologies such as mass spectrometry and atomic force microscopy have revolutionized the field of oral biology. These novel technologies open avenues for a comprehensive characterization of the salivary proteins followed by the evaluation of the physiological functions which could make possible in a near future the development of a new series of synthetic protein for therapeutic propose able to prevent global oral diseases such as periodontal disease and dental caries, the two most prevalent oral diseases in the World.

Keywords Saliva · Proteins · Proteomics · Oral disease · Mass spectrometry · Atomic Force Microscopy

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Introduction

Periodontal disease (i.e., gingivitis, chronic periodontitis) and dental caries are the two most globally prevalent chronic oral pathologies that affect children, youth, adults, and elders (Featherstone 2000; Albandar 2002). In the United States, gingivitis affects 50% of adults, while chronic periodontitis affects an estimated 35% of the adult population (Albandar et al. 1999). In addition, each year over 300,000 patients worldwide are diagnosed with oral cancer (Parkin et al. 1988), representing 2–3% of all malignancies (Parkin et al. 2005). More than 90% of these cases are categorized as oral squamous cell carcinoma (SCC), with high metastasis rates, resulting in high patient mortality (Neville and Day 2002; Parkin et al. 2005).

Recent advances in dental research have enforced the need to gain a more comprehensive understanding of the prevention, treatment, and management of oral diseases. Oral health is an essential component of an individual's well-being because it is very closely related to general health. Throughout the years, oral diseases have been defined as localized oral disturbances, but recent research suggests that they can be considered as general health distal determinants, acting as comorbidities and risk factors for many systemic diseases. For instance, the association between diabetes mellitus and periodontal disease can be considered to be bidirectional: diabetes can be a risk factor for the development of periodontitis (diabetic patients are 2.1–3.0 times more at risk of developing periodontitis; Salvi et al. 1997), while patients with periodontitis are much more likely to develop diabetes (Grossi and Genco 1998; Deshpande et al. 2010). Considering the interconnectedness of oral diseases and general health, we must gain a complete understanding of the pathophysiology of oral diseases within the dynamic, complex oral cavity in

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IV – Análise estatística

A) Virulência

Fosfolipase

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tempo	fosf_trans LSMEAN	LSMEAN Number
24h	1.22226673	1
48h	1.22246890	2
72h	1.23997768	3
Adesion	1.23507108	4

Least Squares Means for effect tempo
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: fosf_trans

i/j	1	2	3	4
1		0.9998	<.0001	<.0001
2	0.9998		<.0001	<.0001
3	<.0001	<.0001		0.2159
4	<.0001	<.0001	0.2159	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tempo	tratame	fosf_trans LSMEAN	LSMEAN Number
24h	Albumina	1.25515185	1
24h	C3b	1.34420302	2
24h	Fibrino	1.29101143	3
24h	Histatin	1.05857493	4
24h	IgA	1.05606118	5
24h	Lacto	1.08740408	6
24h	MucinaI	1.25480297	7
24h	MucinaII	1.27542820	8
24h	Plasma	1.33695170	9
24h	Saliva	1.26307790	10
48h	Albumina	1.23606540	11
48h	C3b	1.32575842	12
48h	Fibrino	1.32927959	13
48h	Histatin	1.08149319	14
48h	IgA	1.08315609	15
48h	Lacto	1.08358044	16
48h	MucinaI	1.24398854	17
48h	MucinaII	1.25899723	18
48h	Plasma	1.32509346	19
48h	Saliva	1.25727660	20
72h	Albumina	1.26143111	21
72h	C3b	1.35800810	22
72h	Fibrino	1.36012909	23
72h	Histatin	1.08149319	24
72h	IgA	1.09837468	25
72h	Lacto	1.09947677	26
72h	MucinaI	1.25209034	27
72h	MucinaII	1.27103942	28
72h	Plasma	1.34813730	29
72h	Saliva	1.26959681	30
Adesion	Albumina	1.25357132	31
Adesion	C3b	1.35589920	32
Adesion	Fibrino	1.35578719	33
Adesion	Histatin	1.07613260	34
Adesion	IgA	1.08947831	35
Adesion	Lacto	1.09031699	36
Adesion	MucinaI	1.24704492	37
Adesion	MucinaII	1.26559312	38
Adesion	Plasma	1.34590430	39
Adesion	Saliva	1.27098284	40

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: fosf_trans

i/j	1	2	3	4	5	6	7	8	9	10
1		<.0001	0.0165	<.0001	<.0001	<.0001	1.0000	0.8648	<.0001	1.0000
2	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
3	0.0165	<.0001		<.0001	<.0001	<.0001	0.0141	0.9985	<.0001	0.2822
4	<.0001	<.0001	<.0001		1.0000	0.1430	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	1.0000		0.0562	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	0.1430	0.0562		<.0001	<.0001	<.0001	<.0001
7	1.0000	<.0001	0.0141	<.0001	<.0001	<.0001		0.8408	<.0001	1.0000
8	0.8648	<.0001	0.9985	<.0001	<.0001	<.0001	0.8408		<.0001	1.0000
9	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
10	1.0000	<.0001	0.2822	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	
11	0.9293	<.0001	<.0001	<.0001	<.0001	<.0001	0.9432	0.0012	<.0001	0.2535
12	<.0001	0.9533	0.0266	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
13	<.0001	0.9984	0.0055	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
14	<.0001	<.0001	<.0001	0.6371	0.3856	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.4678	0.2475	1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.4260	0.2181	1.0000	<.0001	<.0001	<.0001	<.0001
17	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0541	<.0001	0.9291
18	1.0000	<.0001	0.0775	<.0001	<.0001	<.0001	1.0000	0.9912	<.0001	1.0000
19	<.0001	0.9283	0.0349	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
20	1.0000	<.0001	0.0402	<.0001	<.0001	<.0001	1.0000	0.9620	<.0001	1.0000
21	1.0000	<.0001	0.1759	<.0001	<.0001	<.0001	1.0000	0.9995	<.0001	1.0000
22	<.0001	0.9997	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.8081	<.0001
23	<.0001	0.9947	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.6109	<.0001
24	<.0001	<.0001	<.0001	0.6371	0.3856	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.0010	0.0002	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.0005	0.0001	1.0000	<.0001	<.0001	<.0001	<.0001
27	1.0000	<.0001	0.0040	<.0001	<.0001	<.0001	1.0000	0.5945	<.0001	1.0000
28	0.9949	<.0001	0.9311	<.0001	<.0001	<.0001	0.9927	1.0000	<.0001	1.0000
29	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
30	0.9991	<.0001	0.8553	<.0001	<.0001	<.0001	0.9986	1.0000	<.0001	1.0000
31	1.0000	<.0001	0.0081	<.0001	<.0001	<.0001	1.0000	0.7396	<.0001	1.0000
32	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9351	<.0001
33	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9395	<.0001
34	<.0001	<.0001	<.0001	0.9759	0.8778	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	0.0669	0.0234	1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	0.0478	0.0160	1.0000	<.0001	<.0001	<.0001	<.0001
37	1.0000	<.0001	0.0003	<.0001	<.0001	<.0001	1.0000	0.1659	<.0001	0.9941
38	1.0000	<.0001	0.5026	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
39	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: fosf_trans

i/j	11	12	13	14	15	16	17	18	19	20
1	0.9293	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
2	<.0001	0.9533	0.9984	<.0001	<.0001	<.0001	<.0001	<.0001	0.9283	<.0001
3	<.0001	0.0266	0.0055	<.0001	<.0001	<.0001	<.0001	0.0775	0.0349	0.0402
4	<.0001	<.0001	<.0001	0.6371	0.4678	0.4260	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.3856	0.2475	0.2181	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
7	0.9432	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
8	0.0012	<.0001	<.0001	<.0001	<.0001	<.0001	0.0541	0.9912	<.0001	0.9620
9	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
10	0.2535	<.0001	<.0001	<.0001	<.0001	<.0001	0.9291	1.0000	<.0001	1.0000
11		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.6358	<.0001	0.7956
12	<.0001		1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
13	<.0001	1.0000		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
14	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001
17	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		0.9982	<.0001	0.9998
18	0.6358	<.0001	<.0001	<.0001	<.0001	<.0001	0.9982		<.0001	1.0000
19	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
20	0.7956	<.0001	<.0001	<.0001	<.0001	<.0001	0.9998	1.0000	<.0001	
21	0.3917	<.0001	<.0001	<.0001	<.0001	<.0001	0.9780	1.0000	<.0001	1.0000
22	<.0001	0.0388	0.1479	<.0001	<.0001	<.0001	<.0001	<.0001	0.0292	<.0001
23	<.0001	0.0152	0.0684	<.0001	<.0001	<.0001	<.0001	<.0001	0.0111	<.0001
24	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.9865	0.9976	0.9986	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.9664	0.9921	0.9949	<.0001	<.0001	<.0001	<.0001
27	0.9941	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
28	0.0115	<.0001	<.0001	<.0001	<.0001	<.0001	0.2507	1.0000	<.0001	0.9997
29	<.0001	0.6905	0.9386	<.0001	<.0001	<.0001	<.0001	<.0001	0.6244	<.0001
30	0.0222	<.0001	<.0001	<.0001	<.0001	<.0001	0.3694	1.0000	<.0001	1.0000
31	0.9769	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
32	<.0001	0.0896	0.2833	<.0001	<.0001	<.0001	<.0001	<.0001	0.0695	<.0001
33	<.0001	0.0934	0.2922	<.0001	<.0001	<.0001	<.0001	<.0001	0.0726	<.0001
34	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
37	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
38	0.1121	<.0001	<.0001	<.0001	<.0001	<.0001	0.7622	1.0000	<.0001	1.0000
39	<.0001	0.8731	0.9893	<.0001	<.0001	<.0001	<.0001	<.0001	0.8271	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: fosf_trans

i/j	21	22	23	24	25	26	27	28	29	30
1	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.9949	<.0001	0.9991
2	<.0001	0.9997	0.9947	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
3	0.1759	<.0001	<.0001	<.0001	<.0001	<.0001	0.0040	0.9311	<.0001	0.8553
4	<.0001	<.0001	<.0001	0.6371	0.0010	0.0005	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.3856	0.0002	0.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
7	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.9927	<.0001	0.9986
8	0.9995	<.0001	<.0001	<.0001	<.0001	<.0001	0.5945	1.0000	<.0001	1.0000
9	<.0001	0.8081	0.6109	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
10	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
11	0.3917	<.0001	<.0001	<.0001	<.0001	<.0001	0.9941	0.0115	<.0001	0.0222
12	<.0001	0.0388	0.0152	<.0001	<.0001	<.0001	<.0001	<.0001	0.6905	<.0001
13	<.0001	0.1479	0.0684	<.0001	<.0001	<.0001	<.0001	<.0001	0.9386	<.0001
14	<.0001	<.0001	<.0001	1.0000	0.9865	0.9664	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	1.0000	0.9976	0.9921	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	1.0000	0.9986	0.9949	<.0001	<.0001	<.0001	<.0001
17	0.9780	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.2507	<.0001	0.3694
18	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
19	<.0001	0.0292	0.0111	<.0001	<.0001	<.0001	<.0001	<.0001	0.6244	<.0001
20	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.9997	<.0001	1.0000
21		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
22	<.0001		1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
23	<.0001	1.0000		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
24	<.0001	<.0001	<.0001		0.9865	0.9664	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.9865		1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.9664	1.0000		<.0001	<.0001	<.0001	<.0001
27	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		0.9350	<.0001	0.9768
28	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9350		<.0001	1.0000
29	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
30	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9768	1.0000	<.0001	
31	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.9776	<.0001	0.9941
32	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
33	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
34	<.0001	<.0001	<.0001	1.0000	0.7036	0.5938	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
37	0.9992	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.5272	<.0001	0.6736
38	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9998	1.0000	<.0001	1.0000
39	<.0001	1.0000	0.9994	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: fosf_trans

i/j	31	32	33	34	35	36	37	38	39	40
1	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	0.9952
2	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
3	0.0081	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	0.5026	<.0001	0.9288
4	<.0001	<.0001	<.0001	0.9759	0.0669	0.0478	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.8778	0.0234	0.0160	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
7	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	0.9931
8	0.7396	<.0001	<.0001	<.0001	<.0001	<.0001	0.1659	1.0000	<.0001	1.0000
9	<.0001	0.9351	0.9395	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
10	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9941	1.0000	<.0001	1.0000
11	0.9769	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.1121	<.0001	0.0118
12	<.0001	0.0896	0.0934	<.0001	<.0001	<.0001	<.0001	<.0001	0.8731	<.0001
13	<.0001	0.2833	0.2922	<.0001	<.0001	<.0001	<.0001	<.0001	0.9893	<.0001
14	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
17	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.7622	<.0001	0.2548
18	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
19	<.0001	0.0695	0.0726	<.0001	<.0001	<.0001	<.0001	<.0001	0.8271	<.0001
20	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	0.9997
21	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9992	1.0000	<.0001	1.0000
22	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
23	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9994	<.0001
24	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.7036	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.5938	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
27	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.9998	<.0001	0.9373
28	0.9776	<.0001	<.0001	<.0001	<.0001	<.0001	0.5272	1.0000	<.0001	1.0000
29	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
30	0.9941	<.0001	<.0001	<.0001	<.0001	<.0001	0.6736	1.0000	<.0001	1.0000
31		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	0.9786
32	<.0001		1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
33	<.0001	1.0000		<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
34	<.0001	<.0001	<.0001		0.9998	0.9994	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	0.9998		1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	0.9994	1.0000		<.0001	<.0001	<.0001	<.0001
37	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		0.9499	<.0001	0.5329
38	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9499		<.0001	1.0000
39	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001

Hemolisina

The GLM Procedure

Dependent Variable: hemo_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	39	4.00859123	0.10278439	64.23	<.0001
Error	200	0.32007260	0.00160036		
Corrected Total	239	4.32866382			

R-Square	Coeff Var	Root MSE	hemo_trans Mean
0.926057	-11.80434	0.040005	-0.338897

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tempo	3	0.60286537	0.20095512	125.57	<.0001
tratame	9	3.24393008	0.36043668	225.22	<.0001
tempo*tratame	27	0.16179577	0.00599244	3.74	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

tempo	tratame	hemo_trans LSMEAN	LSMEAN Number
24h	Albumina	-0.39181233	1
24h	C3b	-0.48869312	2
24h	Fibrino	-0.50098387	3
24h	Histatin	-0.19840516	4
24h	IgA	-0.18720946	5
24h	Lacto	-0.17585570	6
24h	MucinaI	-0.42455699	7
24h	MucinaII	-0.43576468	8
24h	Plasma	-0.55375531	9
24h	Saliva	-0.42432795	10
48h	Albumina	-0.25077779	11
48h	C3b	-0.38281778	12
48h	Fibrino	-0.34812161	13
48h	Histatin	-0.19245333	14
48h	IgA	-0.14450517	15
48h	Lacto	-0.12644335	16
48h	MucinaI	-0.25928482	17
48h	MucinaII	-0.28473067	18
48h	Plasma	-0.33645282	19
48h	Saliva	-0.27444947	20
72h	Albumina	-0.35715526	21
72h	C3b	-0.45291039	22
72h	Fibrino	-0.45563774	23
72h	Histatin	-0.19245333	24
72h	IgA	-0.15772349	25
72h	Lacto	-0.10822943	26
72h	MucinaI	-0.36520289	27
72h	MucinaII	-0.36680415	28
72h	Plasma	-0.49596478	29
72h	Saliva	-0.36150253	30
Adesion	Albumina	-0.37358752	31
Adesion	C3b	-0.51376507	32
Adesion	Fibrino	-0.50616727	33
Adesion	Histatin	-0.23497631	34
Adesion	IgA	-0.20639242	35
Adesion	Lacto	-0.18460367	36
Adesion	MucinaI	-0.41304187	37
Adesion	MucinaII	-0.42600734	38
Adesion	Plasma	-0.57440558	39
Adesion	Saliva	-0.42793765	40

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hemo_trans

i/j	1	2	3	4	5	6	7	8	9	10
1		0.0216	0.0027	<.0001	<.0001	<.0001	1.0000	0.9976	<.0001	1.0000
2	0.0216		1.0000	<.0001	<.0001	<.0001	0.7038	0.9562	0.6727	0.6962
3	0.0027	1.0000		<.0001	<.0001	<.0001	0.2926	0.6673	0.9578	0.2862
4	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001
7	1.0000	0.7038	0.2926	<.0001	<.0001	<.0001		1.0000	<.0001	1.0000
8	0.9976	0.9562	0.6673	<.0001	<.0001	<.0001	1.0000		0.0005	1.0000
9	<.0001	0.6727	0.9578	<.0001	<.0001	<.0001	<.0001	0.0005		<.0001
10	1.0000	0.6962	0.2862	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	
11	<.0001	<.0001	<.0001	0.9618	0.7224	0.3362	<.0001	<.0001	<.0001	<.0001
12	1.0000	0.0049	0.0005	<.0001	<.0001	<.0001	0.9991	0.9560	<.0001	0.9992
13	0.9978	<.0001	<.0001	<.0001	<.0001	<.0001	0.2923	0.0814	<.0001	0.2987
14	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.9450	0.9986	1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.4310	0.8069	0.9831	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	0.8038	0.4272	0.1381	<.0001	<.0001	<.0001	<.0001
18	0.0040	<.0001	<.0001	0.0966	0.0195	0.0029	<.0001	<.0001	<.0001	<.0001
19	0.9244	<.0001	<.0001	<.0001	<.0001	<.0001	0.0766	0.0147	<.0001	0.0790
20	0.0006	<.0001	<.0001	0.3033	0.0858	0.0165	<.0001	<.0001	<.0001	<.0001
21	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.5909	0.2357	<.0001	0.5990
22	0.7977	1.0000	0.9889	<.0001	<.0001	<.0001	1.0000	1.0000	0.0115	1.0000
23	0.7141	1.0000	0.9958	<.0001	<.0001	<.0001	1.0000	1.0000	0.0178	1.0000
24	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.9995	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.0579	0.2268	0.5829	<.0001	<.0001	<.0001	<.0001
27	1.0000	0.0002	<.0001	<.0001	<.0001	<.0001	0.8439	0.4790	<.0001	0.8495
28	1.0000	0.0002	<.0001	<.0001	<.0001	<.0001	0.8805	0.5355	<.0001	0.8853
29	0.0066	1.0000	1.0000	<.0001	<.0001	<.0001	0.4498	0.8223	0.8798	0.4420
30	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.7389	0.3564	<.0001	0.7461
31	1.0000	0.0009	<.0001	<.0001	<.0001	<.0001	0.9735	0.7661	<.0001	0.9752
32	0.0002	1.0000	1.0000	<.0001	<.0001	<.0001	0.0661	0.2508	0.9996	0.0640
33	0.0010	1.0000	1.0000	<.0001	<.0001	<.0001	0.1705	0.4846	0.9905	0.1661
34	<.0001	<.0001	<.0001	0.9999	0.9899	0.8496	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
37	1.0000	0.3146	0.0783	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
38	1.0000	0.7505	0.3345	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
39	<.0001	0.1044	0.3829	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hemo_trans

i/j	11	12	13	14	15	16	17	18	19	20
1	<.0001	1.0000	0.9978	<.0001	<.0001	<.0001	<.0001	0.0040	0.9244	0.0006
2	<.0001	0.0049	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.0005	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	0.9618	<.0001	<.0001	1.0000	0.9450	0.4310	0.8038	0.0966	<.0001	0.3033
5	0.7224	<.0001	<.0001	1.0000	0.9986	0.8069	0.4272	0.0195	<.0001	0.0858
6	0.3362	<.0001	<.0001	1.0000	1.0000	0.9831	0.1381	0.0029	<.0001	0.0165
7	<.0001	0.9991	0.2923	<.0001	<.0001	<.0001	<.0001	<.0001	0.0766	<.0001
8	<.0001	0.9560	0.0814	<.0001	<.0001	<.0001	<.0001	<.0001	0.0147	<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	<.0001	0.9992	0.2987	<.0001	<.0001	<.0001	<.0001	<.0001	0.0790	<.0001
11		<.0001	0.0201	0.8681	0.0046	0.0001	1.0000	1.0000	0.1049	1.0000
12	<.0001		1.0000	<.0001	<.0001	<.0001	0.0002	0.0179	0.9938	0.0031
13	0.0201	1.0000		<.0001	<.0001	<.0001	0.0695	0.7282	1.0000	0.3749
14	0.8681	<.0001	<.0001		0.9893	0.6400	0.6111	0.0430	<.0001	0.1632
15	0.0046	<.0001	<.0001	0.9893		1.0000	0.0010	<.0001	<.0001	<.0001
16	0.0001	<.0001	<.0001	0.6400	1.0000		<.0001	<.0001	<.0001	<.0001
17	1.0000	0.0002	0.0695	0.6111	0.0010	<.0001		1.0000	0.2724	1.0000
18	1.0000	0.0179	0.7282	0.0430	<.0001	<.0001	1.0000		0.9676	1.0000
19	0.1049	0.9938	1.0000	<.0001	<.0001	<.0001	0.2724	0.9676		0.7713
20	1.0000	0.0031	0.3749	0.1632	<.0001	<.0001	1.0000	1.0000	0.7713	
21	0.0045	1.0000	1.0000	<.0001	<.0001	<.0001	0.0185	0.4155	1.0000	0.1503
22	<.0001	0.4954	0.0059	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007	<.0001
23	<.0001	0.4024	0.0037	<.0001	<.0001	<.0001	<.0001	<.0001	0.0004	<.0001
24	0.8681	<.0001	<.0001	1.0000	0.9893	0.6400	0.6111	0.0430	<.0001	0.1632
25	0.0384	<.0001	<.0001	1.0000	1.0000	1.0000	0.0102	<.0001	<.0001	0.0007
26	<.0001	<.0001	<.0001	0.1255	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
27	0.0010	1.0000	1.0000	<.0001	<.0001	<.0001	0.0049	0.1934	1.0000	0.0534
28	0.0008	1.0000	1.0000	<.0001	<.0001	<.0001	0.0037	0.1617	1.0000	0.0425
29	<.0001	0.0013	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	0.0021	1.0000	1.0000	<.0001	<.0001	<.0001	0.0091	0.2831	1.0000	0.0880
31	0.0002	1.0000	1.0000	<.0001	<.0001	<.0001	0.0011	0.0693	0.9999	0.0151
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
34	1.0000	<.0001	0.0013	0.9987	0.0555	0.0031	1.0000	0.9813	0.0103	0.9997
35	0.9971	<.0001	<.0001	1.0000	0.7748	0.2047	0.9566	0.2423	<.0001	0.5676
36	0.6342	<.0001	<.0001	1.0000	0.9996	0.8718	0.3435	0.0129	<.0001	0.0605
37	<.0001	1.0000	0.6775	<.0001	<.0001	<.0001	<.0001	<.0001	0.2881	<.0001
38	<.0001	0.9982	0.2537	<.0001	<.0001	<.0001	<.0001	<.0001	0.0630	<.0001
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hemo_trans

i/j	21	22	23	24	25	26	27	28	29	30
1	1.0000	0.7977	0.7141	<.0001	<.0001	<.0001	1.0000	1.0000	0.0066	1.0000
2	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	0.0002	0.0002	1.0000	<.0001
3	<.0001	0.9889	0.9958	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
4	<.0001	<.0001	<.0001	1.0000	0.9995	0.0579	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	1.0000	1.0000	0.2268	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000	0.5829	<.0001	<.0001	<.0001	<.0001
7	0.5909	1.0000	1.0000	<.0001	<.0001	<.0001	0.8439	0.8805	0.4498	0.7389
8	0.2357	1.0000	1.0000	<.0001	<.0001	<.0001	0.4790	0.5355	0.8223	0.3564
9	<.0001	0.0115	0.0178	<.0001	<.0001	<.0001	<.0001	<.0001	0.8798	<.0001
10	0.5990	1.0000	1.0000	<.0001	<.0001	<.0001	0.8495	0.8853	0.4420	0.7461
11	0.0045	<.0001	<.0001	0.8681	0.0384	<.0001	0.0010	0.0008	<.0001	0.0021
12	1.0000	0.4954	0.4024	<.0001	<.0001	<.0001	1.0000	1.0000	0.0013	1.0000
13	1.0000	0.0059	0.0037	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
14	<.0001	<.0001	<.0001	1.0000	1.0000	0.1255	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.9893	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.6400	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
17	0.0185	<.0001	<.0001	0.6111	0.0102	<.0001	0.0049	0.0037	<.0001	0.0091
18	0.4155	<.0001	<.0001	0.0430	<.0001	<.0001	0.1934	0.1617	<.0001	0.2831
19	1.0000	0.0007	0.0004	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
20	0.1503	<.0001	<.0001	0.1632	0.0007	<.0001	0.0534	0.0425	<.0001	0.0880
21		0.0257	0.0168	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
22	0.0257		1.0000	<.0001	<.0001	<.0001	0.0807	0.0993	0.9983	0.0487
23	0.0168	1.0000		<.0001	<.0001	<.0001	0.0558	0.0695	0.9995	0.0328
24	<.0001	<.0001	<.0001		1.0000	0.1255	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	1.0000		0.9827	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.1255	0.9827		<.0001	<.0001	<.0001	<.0001
27	1.0000	0.0807	0.0558	<.0001	<.0001	<.0001		1.0000	<.0001	1.0000
28	1.0000	0.0993	0.0695	<.0001	<.0001	<.0001	1.0000		<.0001	1.0000
29	<.0001	0.9983	0.9995	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
30	1.0000	0.0487	0.0328	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	
31	1.0000	0.2188	0.1621	<.0001	<.0001	<.0001	1.0000	1.0000	0.0002	1.0000
32	<.0001	0.8045	0.8725	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
33	<.0001	0.9526	0.9766	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
34	0.0002	<.0001	<.0001	0.9987	0.2701	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000	0.9865	0.0177	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000	0.2940	<.0001	<.0001	<.0001	<.0001
37	0.9159	0.9996	0.9987	<.0001	<.0001	<.0001	0.9897	0.9941	0.1466	0.9692
38	0.5393	1.0000	1.0000	<.0001	<.0001	<.0001	0.8059	0.8476	0.5002	0.6915
39	<.0001	0.0003	0.0004	<.0001	<.0001	<.0001	<.0001	<.0001	0.2398	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hemo_trans

i/j	31	32	33	34	35	36	37	38	39	40
1	1.0000	0.0002	0.0010	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
2	0.0009	1.0000	1.0000	<.0001	<.0001	<.0001	0.3146	0.7505	0.1044	0.8072
3	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	0.0783	0.3345	0.3829	0.3950
4	<.0001	<.0001	<.0001	0.9999	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.9899	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	0.8496	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
7	0.9735	0.0661	0.1705	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
8	0.7661	0.2508	0.4846	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
9	<.0001	0.9996	0.9905	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0001
10	0.9752	0.0640	0.1661	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
11	0.0002	<.0001	<.0001	1.0000	0.9971	0.6342	<.0001	<.0001	<.0001	<.0001
12	1.0000	<.0001	0.0002	<.0001	<.0001	<.0001	1.0000	0.9982	<.0001	0.9961
13	1.0000	<.0001	<.0001	0.0013	<.0001	<.0001	0.6775	0.2537	<.0001	0.2077
14	<.0001	<.0001	<.0001	0.9987	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.0555	0.7748	0.9996	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.0031	0.2047	0.8718	<.0001	<.0001	<.0001	<.0001
17	0.0011	<.0001	<.0001	1.0000	0.9566	0.3435	<.0001	<.0001	<.0001	<.0001
18	0.0693	<.0001	<.0001	0.9813	0.2423	0.0129	<.0001	<.0001	<.0001	<.0001
19	0.9999	<.0001	<.0001	0.0103	<.0001	<.0001	0.2881	0.0630	<.0001	0.0481
20	0.0151	<.0001	<.0001	0.9997	0.5676	0.0605	<.0001	<.0001	<.0001	<.0001
21	1.0000	<.0001	<.0001	0.0002	<.0001	<.0001	0.9159	0.5393	<.0001	0.4714
22	0.2188	0.8045	0.9526	<.0001	<.0001	<.0001	0.9996	1.0000	0.0003	1.0000
23	0.1621	0.8725	0.9766	<.0001	<.0001	<.0001	0.9987	1.0000	0.0004	1.0000
24	<.0001	<.0001	<.0001	0.9987	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.2701	0.9865	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	<.0001	0.0177	0.2940	<.0001	<.0001	<.0001	<.0001
27	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9897	0.8059	<.0001	0.7489
28	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9941	0.8476	<.0001	0.7967
29	0.0002	1.0000	1.0000	<.0001	<.0001	<.0001	0.1466	0.5002	0.2398	0.5687
30	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.9692	0.6915	<.0001	0.6251
31		<.0001	<.0001	<.0001	<.0001	<.0001	0.9997	0.9613	<.0001	0.9391
32	<.0001		1.0000	<.0001	<.0001	<.0001	0.0117	0.0802	0.8104	0.1029
33	<.0001	1.0000		<.0001	<.0001	<.0001	0.0380	0.2001	0.5611	0.2450
34	<.0001	<.0001	<.0001		1.0000	0.9776	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	0.9776	1.0000		<.0001	<.0001	<.0001	<.0001
37	0.9997	0.0117	0.0380	<.0001	<.0001	<.0001		1.0000	<.0001	1.0000
38	0.9613	0.0802	0.2001	<.0001	<.0001	<.0001	1.0000		<.0001	1.0000
39	<.0001	0.8104	0.5611	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001

Proteinase

tempo	prot_trans LSMEAN	LSMEAN Number
24h	-0.27117405	1
48h	-0.24356058	2
72h	-0.23210360	3
Adesion	-0.27616435	4

Least Squares Means for effect tempo
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: prot_trans

i/j	1	2	3	4
1		<.0001	<.0001	0.6315
2	<.0001		0.0336	<.0001
3	<.0001	0.0336		<.0001
4	0.6315	<.0001	<.0001	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

tempo	tratame	prot_trans LSMEAN	LSMEAN Number
tempo	tratame	prot_trans LSMEAN	LSMEAN Number
24h	Albumina	-0.26941851	1
24h	C3b	-0.35082032	2
24h	Fibrino	-0.34740839	3
24h	Histatin	-0.17113211	4
24h	IgA	-0.12165089	5
24h	Lacto	-0.20098831	6
24h	MucinaI	-0.28773093	7
24h	MucinaII	-0.31465637	8
24h	Plasma	-0.32864848	9
24h	Saliva	-0.31928620	10
48h	Albumina	-0.23932743	11
48h	C3b	-0.30436915	12
48h	Fibrino	-0.28437190	13
48h	Histatin	-0.16778878	14
48h	IgA	-0.13318598	15
48h	Lacto	-0.18960876	16
48h	MucinaI	-0.24721737	17
48h	MucinaII	-0.26245869	18
48h	Plasma	-0.35391458	19
48h	Saliva	-0.25336315	20
72h	Albumina	-0.22925201	21
72h	C3b	-0.28147622	22
72h	Fibrino	-0.26768167	23
72h	Histatin	-0.16778878	24
72h	IgA	-0.13644869	25
72h	Lacto	-0.19742284	26
72h	MucinaI	-0.24315624	27
72h	MucinaII	-0.24989355	28
72h	Plasma	-0.30693839	29
72h	Saliva	-0.24097767	30
Adesion	Albumina	-0.22020973	31
Adesion	C3b	-0.38730229	32
Adesion	Fibrino	-0.39642221	33
Adesion	Histatin	-0.16922995	34
Adesion	IgA	-0.12509401	35
Adesion	Lacto	-0.17134726	36
Adesion	MucinaI	-0.27682872	37
Adesion	MucinaII	-0.34425882	38
Adesion	Plasma	-0.38223607	39
Adesion	Saliva	-0.28871448	40

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: prot_trans

i/j	1	2	3	4	5	6	7	8	9	10
1		<.0001	<.0001	<.0001	<.0001	0.0004	1.0000	0.2256	0.0074	0.0872
2	<.0001		1.0000	<.0001	<.0001	<.0001	0.0023	0.7349	0.9998	0.9285
3	<.0001	1.0000		<.0001	<.0001	<.0001	0.0065	0.8914	1.0000	0.9845
4	<.0001	<.0001	<.0001		0.0951	0.9638	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.0951		<.0001	<.0001	<.0001	<.0001	<.0001
6	0.0004	<.0001	<.0001	0.9638	<.0001		<.0001	<.0001	<.0001	<.0001
7	1.0000	0.0023	0.0065	<.0001	<.0001	<.0001		0.9921	0.4471	0.9280
8	0.2256	0.7349	0.8914	<.0001	<.0001	<.0001	0.9921		1.0000	1.0000
9	0.0074	0.9998	1.0000	<.0001	<.0001	<.0001	0.4471	1.0000		1.0000
10	0.0872	0.9285	0.9845	<.0001	<.0001	<.0001	0.9280	1.0000	1.0000	
11	0.9598	<.0001	<.0001	0.0004	<.0001	0.6058	0.1204	<.0001	<.0001	<.0001
12	0.7987	0.1794	0.3281	<.0001	<.0001	<.0001	1.0000	1.0000	0.9988	1.0000
13	1.0000	0.0007	0.0023	<.0001	<.0001	<.0001	1.0000	0.9564	0.2674	0.8005
14	<.0001	<.0001	<.0001	1.0000	0.1906	0.8752	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.6300	1.0000	0.0005	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	1.0000	0.0004	1.0000	<.0001	<.0001	<.0001	<.0001
17	0.9998	<.0001	<.0001	<.0001	<.0001	0.1873	0.4714	0.0005	<.0001	0.0001
18	1.0000	<.0001	<.0001	<.0001	<.0001	0.0037	0.9974	0.0502	0.0008	0.0148
19	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	0.0008	0.5487	0.9974	0.8143
20	1.0000	<.0001	<.0001	<.0001	<.0001	0.0480	0.8264	0.0040	<.0001	0.0009
21	0.4926	<.0001	<.0001	0.0102	<.0001	0.9832	0.0092	<.0001	<.0001	<.0001
22	1.0000	0.0003	0.0009	<.0001	<.0001	<.0001	1.0000	0.8760	0.1555	0.6383
23	1.0000	<.0001	<.0001	<.0001	<.0001	0.0007	1.0000	0.1618	0.0044	0.0580
24	<.0001	<.0001	<.0001	1.0000	0.1906	0.8752	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.8116	1.0000	0.0014	<.0001	<.0001	<.0001	<.0001
26	0.0001	<.0001	<.0001	0.9947	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
27	0.9948	<.0001	<.0001	0.0001	<.0001	0.3749	0.2540	0.0001	<.0001	<.0001
28	1.0000	<.0001	<.0001	<.0001	<.0001	0.1080	0.6366	0.0013	<.0001	0.0003
29	0.6559	0.2860	0.4741	<.0001	<.0001	<.0001	1.0000	1.0000	0.9999	1.0000
30	0.9816	<.0001	<.0001	0.0002	<.0001	0.5034	0.1691	<.0001	<.0001	<.0001
31	0.1010	<.0001	<.0001	0.1040	<.0001	1.0000	0.0005	<.0001	<.0001	<.0001
32	<.0001	0.7170	0.5093	<.0001	<.0001	<.0001	<.0001	<.0001	0.0087	0.0004
33	<.0001	0.2109	0.1055	<.0001	<.0001	<.0001	<.0001	<.0001	0.0005	<.0001
34	<.0001	<.0001	<.0001	1.0000	0.1431	0.9225	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	0.1943	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	0.0906	0.9671	<.0001	<.0001	<.0001	<.0001
37	1.0000	<.0001	0.0002	<.0001	<.0001	<.0001	1.0000	0.6372	0.0550	0.3590
38	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	0.0161	0.9677	1.0000	0.9979
39	<.0001	0.9316	0.8047	<.0001	<.0001	<.0001	<.0001	0.0005	0.0353	0.0024

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: prot_trans

i/j	11	12	13	14	15	16	17	18	19	20
1	0.9598	0.7987	1.0000	<.0001	<.0001	<.0001	0.9998	1.0000	<.0001	1.0000
2	<.0001	0.1794	0.0007	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
3	<.0001	0.3281	0.0023	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
4	0.0004	<.0001	<.0001	1.0000	0.6300	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.1906	1.0000	0.0004	<.0001	<.0001	<.0001	<.0001
6	0.6058	<.0001	<.0001	0.8752	0.0005	1.0000	0.1873	0.0037	<.0001	0.0480
7	0.1204	1.0000	1.0000	<.0001	<.0001	<.0001	0.4714	0.9974	0.0008	0.8264
8	<.0001	1.0000	0.9564	<.0001	<.0001	<.0001	0.0005	0.0502	0.5487	0.0040
9	<.0001	0.9988	0.2674	<.0001	<.0001	<.0001	<.0001	0.0008	0.9974	<.0001
10	<.0001	1.0000	0.8005	<.0001	<.0001	<.0001	0.0001	0.0148	0.8143	0.0009
11		0.0012	0.2336	0.0001	<.0001	0.0902	1.0000	0.9995	<.0001	1.0000
12	0.0012		1.0000	<.0001	<.0001	<.0001	0.0135	0.3894	0.0937	0.0669
13	0.2336	1.0000		<.0001	<.0001	<.0001	0.6778	0.9999	0.0003	0.9414
14	0.0001	<.0001	<.0001		0.8155	0.9999	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.8155		0.0165	<.0001	<.0001	<.0001	<.0001
16	0.0902	<.0001	<.0001	0.9999	0.0165		0.0118	<.0001	<.0001	0.0018
17	1.0000	0.0135	0.6778	<.0001	<.0001	0.0118		1.0000	<.0001	1.0000
18	0.9995	0.3894	0.9999	<.0001	<.0001	<.0001	1.0000		<.0001	1.0000
19	<.0001	0.0937	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
20	1.0000	0.0669	0.9414	<.0001	<.0001	0.0018	1.0000	1.0000	<.0001	
21	1.0000	<.0001	0.0236	0.0038	<.0001	0.5248	1.0000	0.8749	<.0001	0.9989
22	0.3760	0.9996	1.0000	<.0001	<.0001	<.0001	0.8313	1.0000	<.0001	0.9845
23	0.9824	0.7052	1.0000	<.0001	<.0001	<.0001	1.0000	1.0000	<.0001	1.0000
24	0.0001	<.0001	<.0001	1.0000	0.8155	0.9999	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.9335	1.0000	0.0394	<.0001	<.0001	<.0001	<.0001
26	0.3897	<.0001	<.0001	0.9672	0.0016	1.0000	0.0886	0.0012	<.0001	0.0189
27	1.0000	0.0041	0.4295	<.0001	<.0001	0.0357	1.0000	1.0000	<.0001	1.0000
28	1.0000	0.0280	0.8213	<.0001	<.0001	0.0054	1.0000	1.0000	<.0001	1.0000
29	0.0005	1.0000	0.9997	<.0001	<.0001	<.0001	0.0064	0.2582	0.1618	0.0355
30	1.0000	0.0020	0.3099	<.0001	<.0001	0.0614	1.0000	0.9999	<.0001	1.0000
31	1.0000	<.0001	0.0016	0.0475	<.0001	0.9502	0.9917	0.3704	<.0001	0.8770
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.8680	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.3563	<.0001
34	0.0002	<.0001	<.0001	1.0000	0.7415	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	0.3462	1.0000	0.0014	<.0001	<.0001	<.0001	<.0001
36	0.0004	<.0001	<.0001	1.0000	0.6168	1.0000	<.0001	<.0001	<.0001	<.0001
37	0.6570	0.9887	1.0000	<.0001	<.0001	<.0001	0.9675	1.0000	<.0001	0.9994
38	<.0001	0.5096	0.0061	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9827	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: prot_trans

i/j	21	22	23	24	25	26	27	28	29	30
1	0.4926	1.0000	1.0000	<.0001	<.0001	0.0001	0.9948	1.0000	0.6559	0.9816
2	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.2860	<.0001
3	<.0001	0.0009	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.4741	<.0001
4	0.0102	<.0001	<.0001	1.0000	0.8116	0.9947	0.0001	<.0001	<.0001	0.0002
5	<.0001	<.0001	<.0001	0.1906	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001
6	0.9832	<.0001	0.0007	0.8752	0.0014	1.0000	0.3749	0.1080	<.0001	0.5034
7	0.0092	1.0000	1.0000	<.0001	<.0001	<.0001	0.2540	0.6366	1.0000	0.1691
8	<.0001	0.8760	0.1618	<.0001	<.0001	<.0001	0.0001	0.0013	1.0000	<.0001
9	<.0001	0.1555	0.0044	<.0001	<.0001	<.0001	<.0001	<.0001	0.9999	<.0001
10	<.0001	0.6383	0.0580	<.0001	<.0001	<.0001	<.0001	0.0003	1.0000	<.0001
11	1.0000	0.3760	0.9824	0.0001	<.0001	0.3897	1.0000	1.0000	0.0005	1.0000
12	<.0001	0.9996	0.7052	<.0001	<.0001	<.0001	0.0041	0.0280	1.0000	0.0020
13	0.0236	1.0000	1.0000	<.0001	<.0001	<.0001	0.4295	0.8213	0.9997	0.3099
14	0.0038	<.0001	<.0001	1.0000	0.9335	0.9672	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.8155	1.0000	0.0016	<.0001	<.0001	<.0001	<.0001
16	0.5248	<.0001	<.0001	0.9999	0.0394	1.0000	0.0357	0.0054	<.0001	0.0614
17	1.0000	0.8313	1.0000	<.0001	<.0001	0.0886	1.0000	1.0000	0.0064	1.0000
18	0.8749	1.0000	1.0000	<.0001	<.0001	0.0012	1.0000	1.0000	0.2582	0.9999
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.1618	<.0001
20	0.9989	0.9845	1.0000	<.0001	<.0001	0.0189	1.0000	1.0000	0.0355	1.0000
21		0.0498	0.6002	0.0038	<.0001	0.9205	1.0000	1.0000	<.0001	1.0000
22	0.0498		1.0000	<.0001	<.0001	<.0001	0.6070	0.9272	0.9970	0.4723
23	0.6002	1.0000		<.0001	<.0001	0.0002	0.9985	1.0000	0.5488	0.9931
24	0.0038	<.0001	<.0001		0.9335	0.9672	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.9335		0.0044	<.0001	<.0001	<.0001	<.0001
26	0.9205	<.0001	0.0002	0.9672	0.0044		0.2058	0.0469	<.0001	0.3019
27	1.0000	0.6070	0.9985	<.0001	<.0001	0.2058		1.0000	0.0018	1.0000
28	1.0000	0.9272	1.0000	<.0001	<.0001	0.0469	1.0000		0.0139	1.0000
29	<.0001	0.9970	0.5488	<.0001	<.0001	<.0001	0.0018	0.0139		0.0009
30	1.0000	0.4723	0.9931	<.0001	<.0001	0.3019	1.0000	1.0000	0.0009	
31	1.0000	0.0040	0.1463	0.0475	<.0001	0.9997	0.9996	0.9665	<.0001	1.0000
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
34	0.0058	<.0001	<.0001	1.0000	0.8904	0.9839	<.0001	<.0001	<.0001	0.0001
35	<.0001	<.0001	<.0001	0.3462	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001
36	0.0109	<.0001	<.0001	1.0000	0.8013	0.9954	0.0001	<.0001	<.0001	0.0003
37	0.1432	1.0000	1.0000	<.0001	<.0001	<.0001	0.8566	0.9921	0.9595	0.7521
38	<.0001	0.0025	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.6679	<.0001
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: prot_trans

i/j	31	32	33	34	35	36	37	38	39	40
1	0.1010	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	1.0000
2	<.0001	0.7170	0.2109	<.0001	<.0001	<.0001	<.0001	1.0000	0.9316	0.0031
3	<.0001	0.5093	0.1055	<.0001	<.0001	<.0001	0.0002	1.0000	0.8047	0.0086
4	0.1040	<.0001	<.0001	1.0000	0.1943	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.1431	1.0000	0.0906	<.0001	<.0001	<.0001	<.0001
6	1.0000	<.0001	<.0001	0.9225	<.0001	0.9671	<.0001	<.0001	<.0001	<.0001
7	0.0005	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0161	<.0001	1.0000
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.6372	0.9677	0.0005	0.9958
9	<.0001	0.0087	0.0005	<.0001	<.0001	<.0001	0.0550	1.0000	0.0353	0.5069
10	<.0001	0.0004	<.0001	<.0001	<.0001	<.0001	0.3590	0.9979	0.0024	0.9508
11	1.0000	<.0001	<.0001	0.0002	<.0001	0.0004	0.6570	<.0001	<.0001	0.0971
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9887	0.5096	<.0001	1.0000
13	0.0016	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0061	<.0001	1.0000
14	0.0475	<.0001	<.0001	1.0000	0.3462	1.0000	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	0.7415	1.0000	0.6168	<.0001	<.0001	<.0001	<.0001
16	0.9502	<.0001	<.0001	1.0000	0.0014	1.0000	<.0001	<.0001	<.0001	<.0001
17	0.9917	<.0001	<.0001	<.0001	<.0001	<.0001	0.9675	<.0001	<.0001	0.4130
18	0.3704	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	0.9948
19	<.0001	0.8680	0.3563	<.0001	<.0001	<.0001	<.0001	1.0000	0.9827	0.0011
20	0.8770	<.0001	<.0001	<.0001	<.0001	<.0001	0.9994	<.0001	<.0001	0.7785
21	1.0000	<.0001	<.0001	0.0058	<.0001	0.0109	0.1432	<.0001	<.0001	0.0069
22	0.0040	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0025	<.0001	1.0000
23	0.1463	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	0.9999
24	0.0475	<.0001	<.0001	1.0000	0.3462	1.0000	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	0.8904	1.0000	0.8013	<.0001	<.0001	<.0001	<.0001
26	0.9997	<.0001	<.0001	0.9839	<.0001	0.9954	<.0001	<.0001	<.0001	<.0001
27	0.9996	<.0001	<.0001	<.0001	<.0001	0.0001	0.8566	<.0001	<.0001	0.2126
28	0.9665	<.0001	<.0001	<.0001	<.0001	<.0001	0.9921	<.0001	<.0001	0.5759
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9595	0.6679	<.0001	1.0000
30	1.0000	<.0001	<.0001	0.0001	<.0001	0.0003	0.7521	<.0001	<.0001	0.1385
31		<.0001	<.0001	0.0673	<.0001	0.1090	0.0157	<.0001	<.0001	0.0004
32	<.0001		1.0000	<.0001	<.0001	<.0001	<.0001	0.3279	1.0000	<.0001
33	<.0001	1.0000		<.0001	<.0001	<.0001	<.0001	0.0506	1.0000	<.0001
34	0.0673	<.0001	<.0001		0.2740	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	0.2740		0.1864	<.0001	<.0001	<.0001	<.0001
36	0.1090	<.0001	<.0001	1.0000	0.1864		<.0001	<.0001	<.0001	<.0001
37	0.0157	<.0001	<.0001	<.0001	<.0001	<.0001		0.0005	<.0001	1.0000
38	<.0001	0.3279	0.0506	<.0001	<.0001	<.0001	0.0005		0.6281	0.0210
39	<.0001	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	0.6281		<.0001

Lipase

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tempo	lip_trans LSMEAN	LSMEAN Number
24h	2.03732880	1
48h	1.54390257	2
72h	1.83312562	3
Adesion	2.00495233	4

Least Squares Means for effect tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: lip_trans

i/j	1	2	3	4
1		<.0001	<.0001	0.3058
2	<.0001		<.0001	<.0001
3	<.0001	<.0001		<.0001
4	0.3058	<.0001	<.0001	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tempo	tratame	lip_trans LSMEAN	LSMEAN Number
24h	Albumina	2.13925487	1
24h	C3b	2.39153139	2
24h	Fibrino	2.64586955	3
24h	Histatin	1.50537907	4
24h	IgA	1.53782560	5
24h	Lacto	1.53892723	6
24h	MucinaI	2.14539377	7
24h	MucinaII	1.99479920	8
24h	Plasma	2.46386128	9
24h	Saliva	2.01044604	10
48h	Albumina	1.83195075	11
48h	C3b	1.66387755	12
48h	Fibrino	1.78757508	13
48h	Histatin	1.16968847	14
48h	IgA	1.10015885	15
48h	Lacto	1.06699545	16
48h	MucinaI	1.60151721	17
48h	MucinaII	1.59420956	18
48h	Plasma	1.83407419	19
48h	Saliva	1.78897855	20
72h	Albumina	1.74580651	21
72h	C3b	1.96352066	22
72h	Fibrino	2.43684395	23
72h	Histatin	1.16968847	24
72h	IgA	1.47329740	25
72h	Lacto	1.36781722	26
72h	MucinaI	1.94291696	27
72h	MucinaII	2.01442244	28
72h	Plasma	2.24091484	29
72h	Saliva	1.97602773	30
Adesion	Albumina	2.11330296	31
Adesion	C3b	2.32723134	32
Adesion	Fibrino	2.55567246	33
Adesion	Histatin	1.47870742	34
Adesion	IgA	1.51448960	35
Adesion	Lacto	1.50360647	36
Adesion	MucinaI	2.27267280	37
Adesion	MucinaII	1.97641981	38
Adesion	Plasma	2.39347059	39
Adesion	Saliva	1.91394981	40

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: lip_trans

i/j	1	2	3	4	5	6	7	8	9	10
1		0.0154	<.0001	<.0001	<.0001	<.0001	1.0000	0.9013	<.0001	0.9763
2	0.0154		0.0135	<.0001	<.0001	<.0001	0.0225	<.0001	1.0000	<.0001
3	<.0001	0.0135		<.0001	<.0001	<.0001	<.0001	<.0001	0.4485	<.0001
4	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001
7	1.0000	0.0225	<.0001	<.0001	<.0001	<.0001		0.8497	0.0001	0.9557
8	0.9013	<.0001	<.0001	<.0001	<.0001	<.0001	0.8497		<.0001	1.0000
9	<.0001	1.0000	0.4485	<.0001	<.0001	<.0001	0.0001	<.0001		<.0001
10	0.9763	<.0001	<.0001	<.0001	<.0001	<.0001	0.9557	1.0000	<.0001	
11	0.0003	<.0001	<.0001	<.0001	0.0008	0.0009	0.0002	0.7108	<.0001	0.4963
12	<.0001	<.0001	<.0001	0.7648	0.9827	0.9848	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	0.0020	0.0180	0.0193	<.0001	0.1757	<.0001	0.0832
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	0.9999	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
18	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
19	0.0011	<.0001	<.0001	0.0002	0.0020	0.0022	0.0007	0.8234	<.0001	0.6399
20	<.0001	<.0001	<.0001	0.0018	0.0165	0.0177	<.0001	0.1868	<.0001	0.0894
21	<.0001	<.0001	<.0001	0.0316	0.1699	0.1784	<.0001	0.0189	<.0001	0.0069
22	0.5346	<.0001	<.0001	<.0001	<.0001	<.0001	0.4503	1.0000	<.0001	1.0000
23	0.0007	1.0000	0.1622	<.0001	<.0001	<.0001	0.0010	<.0001	1.0000	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.9437	0.6145	0.5992	<.0001	<.0001	<.0001	<.0001
27	0.2750	<.0001	<.0001	<.0001	<.0001	<.0001	0.2153	1.0000	<.0001	1.0000
28	0.9850	<.0001	<.0001	<.0001	<.0001	<.0001	0.9702	1.0000	<.0001	1.0000
29	0.9996	0.8495	<.0001	<.0001	<.0001	<.0001	0.9999	0.0225	0.0829	0.0556
30	0.7059	<.0001	<.0001	<.0001	<.0001	<.0001	0.6233	1.0000	<.0001	1.0000
31	1.0000	0.0027	<.0001	<.0001	<.0001	<.0001	1.0000	0.9935	<.0001	0.9995
32	0.3712	1.0000	0.0001	<.0001	<.0001	<.0001	0.4508	<.0001	0.9482	0.0001
33	<.0001	0.6939	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
34	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
37	0.9617	0.9931	<.0001	<.0001	<.0001	<.0001	0.9801	0.0028	0.3324	0.0081
38	0.7109	<.0001	<.0001	<.0001	<.0001	<.0001	0.6287	1.0000	<.0001	1.0000
39	0.0136	1.0000	0.0153	<.0001	<.0001	<.0001	0.0200	<.0001	1.0000	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: lip_trans

i/j	11	12	13	14	15	16	17	18	19	20
1	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0011	<.0001
2	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	0.7648	0.0020	<.0001	<.0001	<.0001	0.9999	1.0000	0.0002	0.0018
5	0.0008	0.9827	0.0180	<.0001	<.0001	<.0001	1.0000	1.0000	0.0020	0.0165
6	0.0009	0.9848	0.0193	<.0001	<.0001	<.0001	1.0000	1.0000	0.0022	0.0177
7	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007	<.0001
8	0.7108	<.0001	0.1757	<.0001	<.0001	<.0001	<.0001	<.0001	0.8234	0.1868
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	0.4963	<.0001	0.0832	<.0001	<.0001	<.0001	<.0001	<.0001	0.6399	0.0894
11		0.6411	1.0000	<.0001	<.0001	<.0001	0.0557	0.0370	1.0000	1.0000
12	0.6411		0.9870	<.0001	<.0001	<.0001	1.0000	1.0000	0.7181	0.9846
13	1.0000	0.9870		<.0001	<.0001	<.0001	0.3954	0.3074	1.0000	1.0000
14	<.0001	<.0001	<.0001		1.0000	0.9996	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.9996	1.0000		<.0001	<.0001	<.0001	<.0001
17	0.0557	1.0000	0.3954	<.0001	<.0001	<.0001		1.0000	0.0882	0.3776
18	0.0370	1.0000	0.3074	<.0001	<.0001	<.0001	1.0000		0.0612	0.2918
19	1.0000	0.7181	1.0000	<.0001	<.0001	<.0001	0.0882	0.0612		1.0000
20	1.0000	0.9846	1.0000	<.0001	<.0001	<.0001	0.3776	0.2918	1.0000	
21	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	0.9025	0.8400	1.0000	1.0000
22	0.9682	0.0006	0.5317	<.0001	<.0001	<.0001	<.0001	<.0001	0.9874	0.5513
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	1.0000	1.0000	0.9996	<.0001	<.0001	<.0001	<.0001
25	<.0001	0.3396	0.0002	0.0004	<.0001	<.0001	0.9778	0.9909	<.0001	0.0002
26	<.0001	0.0007	<.0001	0.2566	0.0056	0.0005	0.0465	0.0692	<.0001	<.0001
27	0.9979	0.0026	0.8010	<.0001	<.0001	<.0001	<.0001	<.0001	0.9994	0.8161
28	0.4423	<.0001	0.0676	<.0001	<.0001	<.0001	<.0001	<.0001	0.5874	0.0728
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	0.9041	0.0002	0.3653	<.0001	<.0001	<.0001	<.0001	<.0001	0.9540	0.3828
31	0.0022	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0062	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
34	<.0001	0.4068	0.0003	0.0003	<.0001	<.0001	0.9884	0.9958	<.0001	0.0002
35	0.0001	0.8609	0.0039	<.0001	<.0001	<.0001	1.0000	1.0000	0.0004	0.0035
36	<.0001	0.7433	0.0018	<.0001	<.0001	<.0001	0.9998	1.0000	0.0002	0.0016
37	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
38	0.9012	0.0002	0.3605	<.0001	<.0001	<.0001	<.0001	<.0001	0.9523	0.3779
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: lip_trans

i/j	21	22	23	24	25	26	27	28	29	30
1	<.0001	0.5346	0.0007	<.0001	<.0001	<.0001	0.2750	0.9850	0.9996	0.7059
2	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.8495	<.0001
3	<.0001	<.0001	0.1622	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	0.0316	<.0001	<.0001	<.0001	1.0000	0.9437	<.0001	<.0001	<.0001	<.0001
5	0.1699	<.0001	<.0001	<.0001	1.0000	0.6145	<.0001	<.0001	<.0001	<.0001
6	0.1784	<.0001	<.0001	<.0001	1.0000	0.5992	<.0001	<.0001	<.0001	<.0001
7	<.0001	0.4503	0.0010	<.0001	<.0001	<.0001	0.2153	0.9702	0.9999	0.6233
8	0.0189	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	0.0225	1.0000
9	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.0829	<.0001
10	0.0069	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	0.0556	1.0000
11	1.0000	0.9682	<.0001	<.0001	<.0001	<.0001	0.9979	0.4423	<.0001	0.9041
12	1.0000	0.0006	<.0001	<.0001	0.3396	0.0007	0.0026	<.0001	<.0001	0.0002
13	1.0000	0.5317	<.0001	<.0001	0.0002	<.0001	0.8010	0.0676	<.0001	0.3653
14	<.0001	<.0001	<.0001	1.0000	0.0004	0.2566	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	1.0000	<.0001	0.0056	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	0.9996	<.0001	0.0005	<.0001	<.0001	<.0001	<.0001
17	0.9025	<.0001	<.0001	<.0001	0.9778	0.0465	<.0001	<.0001	<.0001	<.0001
18	0.8400	<.0001	<.0001	<.0001	0.9909	0.0692	<.0001	<.0001	<.0001	<.0001
19	1.0000	0.9874	<.0001	<.0001	<.0001	<.0001	0.9994	0.5874	<.0001	0.9540
20	1.0000	0.5513	<.0001	<.0001	0.0002	<.0001	0.8161	0.0728	<.0001	0.3828
21		0.1078	<.0001	<.0001	0.0040	<.0001	0.2670	0.0053	<.0001	0.0563
22	0.1078		<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	0.0029	1.0000
23	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	0.2793	<.0001
24	<.0001	<.0001	<.0001		0.0004	0.2566	<.0001	<.0001	<.0001	<.0001
25	0.0040	<.0001	<.0001	0.0004		0.9992	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.2566	0.9992		<.0001	<.0001	<.0001	<.0001
27	0.2670	1.0000	<.0001	<.0001	<.0001	<.0001		1.0000	0.0006	1.0000
28	0.0053	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000		0.0689	1.0000
29	<.0001	0.0029	0.2793	<.0001	<.0001	<.0001	0.0006	0.0689		0.0068
30	0.0563	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	0.0068	
31	<.0001	0.8573	<.0001	<.0001	<.0001	<.0001	0.6092	0.9998	0.9793	0.9451
32	<.0001	<.0001	0.9984	<.0001	<.0001	<.0001	<.0001	0.0002	1.0000	<.0001
33	<.0001	<.0001	0.9932	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001
34	0.0059	<.0001	<.0001	0.0003	1.0000	0.9980	<.0001	<.0001	<.0001	<.0001
35	0.0531	<.0001	<.0001	<.0001	1.0000	0.8842	<.0001	<.0001	<.0001	<.0001
36	0.0285	<.0001	<.0001	<.0001	1.0000	0.9520	<.0001	<.0001	<.0001	<.0001
37	<.0001	0.0003	0.6935	<.0001	<.0001	<.0001	<.0001	0.0105	1.0000	0.0007
38	0.0552	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	0.0070	1.0000
39	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.8305	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect tempo*tratame
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: lip_trans

i/j	31	32	33	34	35	36	37	38	39	40
1	1.0000	0.3712	<.0001	<.0001	<.0001	<.0001	0.9617	0.7109	0.0136	0.0733
2	0.0027	1.0000	0.6939	<.0001	<.0001	<.0001	0.9931	<.0001	1.0000	<.0001
3	<.0001	0.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	0.0153	<.0001
4	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
7	1.0000	0.4508	<.0001	<.0001	<.0001	<.0001	0.9801	0.6287	0.0200	0.0527
8	0.9935	<.0001	<.0001	<.0001	<.0001	<.0001	0.0028	1.0000	<.0001	1.0000
9	<.0001	0.9482	1.0000	<.0001	<.0001	<.0001	0.3324	<.0001	1.0000	<.0001
10	0.9995	0.0001	<.0001	<.0001	<.0001	<.0001	0.0081	1.0000	<.0001	0.9999
11	0.0022	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	0.9012	<.0001	1.0000
12	<.0001	<.0001	<.0001	0.4068	0.8609	0.7433	<.0001	0.0002	<.0001	0.0177
13	<.0001	<.0001	<.0001	0.0003	0.0039	0.0018	<.0001	0.3605	<.0001	0.9820
14	<.0001	<.0001	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	0.9884	1.0000	0.9998	<.0001	<.0001	<.0001	0.0002
18	<.0001	<.0001	<.0001	0.9958	1.0000	1.0000	<.0001	<.0001	<.0001	0.0001
19	0.0062	<.0001	<.0001	<.0001	0.0004	0.0002	<.0001	0.9523	<.0001	1.0000
20	<.0001	<.0001	<.0001	0.0002	0.0035	0.0016	<.0001	0.3779	<.0001	0.9848
21	<.0001	<.0001	<.0001	0.0059	0.0531	0.0285	<.0001	0.0552	<.0001	0.6402
22	0.8573	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	1.0000	<.0001	1.0000
23	<.0001	0.9984	0.9932	<.0001	<.0001	<.0001	0.6935	<.0001	1.0000	<.0001
24	<.0001	<.0001	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	0.9980	0.8842	0.9520	<.0001	<.0001	<.0001	<.0001
27	0.6092	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	1.0000
28	0.9998	0.0002	<.0001	<.0001	<.0001	<.0001	0.0105	1.0000	<.0001	0.9997
29	0.9793	1.0000	0.0002	<.0001	<.0001	<.0001	1.0000	0.0070	0.8305	<.0001
30	0.9451	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007	1.0000	<.0001	1.0000
31		0.1294	<.0001	<.0001	<.0001	<.0001	0.7543	0.9470	0.0024	0.2445
32	0.1294		0.0620	<.0001	<.0001	<.0001	1.0000	<.0001	1.0000	<.0001
33	<.0001	0.0620		<.0001	<.0001	<.0001	0.0019	<.0001	0.7191	<.0001
34	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001
37	0.7543	1.0000	0.0019	<.0001	<.0001	<.0001		0.0007	0.9910	<.0001
38	0.9470	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007		<.0001	1.0000
39	0.0024	1.0000	0.7191	<.0001	<.0001	<.0001	0.9910	<.0001		<.0001

B) Energia livre de superfície

The ANOVA Procedure

Dependent Variable: els

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	19	36.9747500	1.9460395		
Corrected Total	21	257.6571091			

R-Square	Coeff Var	Root MSE	els Mean
0.856496	3.467888	1.395005	40.22636

Source	DF	Anova SS	Mean Square	F Value	Pr > F
trat	2	220.6823591	110.3411795	56.70	<.0001
Model	2	220.6823591	110.3411795	56.70	<.0001

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for els

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	1.946039
Critical Value of Studentized Range	3.59274

Comparisons significant at the 0.05 level are indicated by ***.

trat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Sal_plas - controle	3.2625	1.4905	5.0345	***
Sal_plas - Saliva	8.0200	6.1061	9.9339	***
controle - Sal_plas	-3.2625	-5.0345	-1.4905	***
controle - Saliva	4.7575	2.8436	6.6714	***
Saliva - Sal_plas	-8.0200	-9.9339	-6.1061	***
Saliva - controle	-4.7575	-6.6714	-2.8436	***

The ANOVA Procedure

Dependent Variable: polar

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	18	27.2608548	1.5144919		
Corrected Total	20	487.2132952			

R-Square	Coeff Var	Root MSE	polar Mean
0.944047	25.87464	1.230647	4.756190

Source	DF	Anova SS	Mean Square	F Value	Pr > F
trat	2	459.9524405	229.9762202	151.85	<.0001
Model	2	459.9524405	229.9762202	151.85	<.0001

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for polar

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	1.514492
Critical Value of Studentized Range	3.60930

Comparisons significant at the 0.05 level are indicated by ***.

trat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Sal_plas - Saliva	8.8908	7.1946	10.5871	***
Sal_plas - controle	10.1732	8.5477	11.7987	***
Saliva - Sal_plas	-8.8908	-10.5871	-7.1946	***
Saliva - controle	1.2824	-0.4650	3.0298	
controle - Sal_plas	-10.1732	-11.7987	-8.5477	***
controle - Saliva	-1.2824	-3.0298	0.4650	

The ANOVA Procedure

Dependent Variable: apolar

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	244.1758333	122.0879167	116.84	<.0001
Error	21	21.9437500	1.0449405		
Corrected Total	23	266.1195833			

R-Square	Coeff Var	Root MSE	apolar Mean
0.917542	2.881193	1.022223	35.47917

Source	DF	Anova SS	Mean Square	F Value	Pr > F
trat	2	244.1758333	122.0879167	116.84	<.0001

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for apolar

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	1.04494
Critical Value of Studentized Range	3.56463
Minimum Significant Difference	1.2883

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	trat
A	39.9750	8	controle
B	33.5500	8	Saliva
B			
B	32.9125	8	Sal_plas

C) Confocal

The GLM Procedure

Dependent Variable: av_dff_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	324	1.65778612	0.00511662		
Corrected Total	359	2.80041560			

R-Square	Coeff Var	Root MSE	av_dff_trans Mean
0.408021	7.567198	0.071531	0.945272

Source	DF	Type III SS	Mean Square	F Value	Pr > F
proteina	8	0.25937024	0.03242128	6.34	<.0001
tempo	3	0.39250686	0.13083562	25.57	<.0001
proteina*tempo	24	0.49075238	0.02044802	4.00	<.0001
Model	35	1.14262948	0.03264656	6.38	<.0001

The GLM Procedure

Dependent Variable: av_thick_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	324	42.17303987	0.13016370		
Corrected Total	359	72.02504692			

R-Square	Coeff Var	Root MSE	av_thick_trans Mean
0.414467	9.084387	0.360782	3.971452

Source	DF	Type III SS	Mean Square	F Value	Pr > F
proteina	8	7.17229484	0.89653685	6.89	<.0001
tempo	3	9.94162742	3.31387581	25.46	<.0001
proteina*tempo	24	12.73808479	0.53075353	4.08	<.0001
Model	35	29.85200705	0.85291449	6.55	<.0001

The GLM Procedure

Dependent Variable: biom_trans

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	324	1.85512143	0.00572568		
Corrected Total	359	3.11668597			

R-Square	Coeff Var	Root MSE	biom_trans Mean
0.404778	6.199525	0.075668	1.220549

Source	DF	Type III SS	Mean Square	F Value	Pr > F
proteina	8	0.28912598	0.03614075	6.31	<.0001
tempo	3	0.42419789	0.14139930	24.70	<.0001
proteina*tempo	24	0.54824068	0.02284336	3.99	<.0001
Model	35	1.26156455	0.03604470	6.30	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

proteina	tempo	av_dff_trans LSMEAN	LSMEAN Number
albumina	24h	0.99365815	1
albumina	48h	0.99176018	2
albumina	72h	0.91832470	3
albumina	ades	0.95683095	4
c3b	24h	0.87207029	5
c3b	48h	0.91988303	6
c3b	72h	0.98008601	7
c3b	ades	0.86872513	8
fibrino	24h	1.04997777	9
fibrino	48h	0.95896694	10
fibrino	72h	0.96008945	11
fibrino	ades	0.97038239	12
his	24h	1.06200118	13
his	48h	0.98799704	14
his	72h	0.95143419	15
his	ades	0.82116193	16
iga	24h	0.87647549	17
iga	48h	0.93443679	18
iga	72h	0.96759255	19
iga	ades	0.86926527	20
lacto	24h	0.97210661	21
lacto	48h	0.91615833	22
lacto	72h	0.90657906	23
lacto	ades	0.85521734	24
muc_1	24h	1.01160556	25
muc_1	48h	1.03774887	26
muc_1	72h	0.96177550	27
muc_1	ades	0.88586491	28
muc_2	24h	1.01498138	29
muc_2	48h	0.97202371	30
muc_2	72h	0.94223528	31
muc_2	ades	0.89211649	32
saliva	24h	0.91918674	33
saliva	48h	0.96144004	34
saliva	72h	0.98122487	35
saliva	ades	0.88839725	36

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_dff_trans

i/j	1	2	3	4	5	6	7	8	9
1		1.0000	0.9177	1.0000	0.0635	0.9350	1.0000	0.0450	0.9989
2	1.0000		0.9384	1.0000	0.0766	0.9523	1.0000	0.0548	0.9981
3	0.9177	0.9384		1.0000	1.0000	1.0000	0.9946	0.9999	0.0215
4	1.0000	1.0000	1.0000		0.7529	1.0000	1.0000	0.6735	0.5446
5	0.0635	0.0766	1.0000	0.7529		1.0000	0.2136	1.0000	<.0001
6	0.9350	0.9523	1.0000	1.0000	1.0000		0.9965	0.9998	0.0257
7	1.0000	1.0000	0.9946	1.0000	0.2136	0.9965		0.1631	0.9666
8	0.0450	0.0548	0.9999	0.6735	1.0000	0.9998	0.1631		<.0001
9	0.9989	0.9981	0.0215	0.5446	<.0001	0.0257	0.9666	<.0001	
10	1.0000	1.0000	1.0000	1.0000	0.7031	1.0000	1.0000	0.6196	0.5999
11	1.0000	1.0000	1.0000	1.0000	0.6757	1.0000	1.0000	0.5907	0.6286
12	1.0000	1.0000	0.9998	1.0000	0.4141	0.9999	1.0000	0.3367	0.8557
13	0.9752	0.9643	0.0050	0.2644	<.0001	0.0061	0.8130	<.0001	1.0000
14	1.0000	1.0000	0.9679	1.0000	0.1093	0.9764	1.0000	0.0798	0.9943
15	1.0000	1.0000	1.0000	1.0000	0.8597	1.0000	1.0000	0.7970	0.4085
16	<.0001	0.0001	0.4423	0.0134	0.9999	0.4043	0.0006	1.0000	<.0001
17	0.0974	0.1159	1.0000	0.8424	1.0000	1.0000	0.2952	1.0000	<.0001
18	0.9974	0.9985	1.0000	1.0000	0.9937	1.0000	1.0000	0.9858	0.1133
19	1.0000	1.0000	0.9999	1.0000	0.4835	1.0000	1.0000	0.4008	0.8036
20	0.0476	0.0579	0.9999	0.6868	1.0000	0.9999	0.1706	1.0000	<.0001
21	1.0000	1.0000	0.9996	1.0000	0.3733	0.9998	1.0000	0.2999	0.8834
22	0.8889	0.9144	1.0000	1.0000	1.0000	1.0000	0.9907	1.0000	0.0167
23	0.6987	0.7433	1.0000	0.9999	1.0000	1.0000	0.9376	1.0000	0.0051
24	0.0096	0.0121	0.9924	0.3376	1.0000	0.9889	0.0453	1.0000	<.0001
25	1.0000	1.0000	0.5411	0.9994	0.0084	0.5815	1.0000	0.0055	1.0000
26	1.0000	1.0000	0.0786	0.8321	0.0002	0.0913	0.9984	0.0001	1.0000
27	1.0000	1.0000	1.0000	1.0000	0.6333	1.0000	1.0000	0.5471	0.6711
28	0.2173	0.2508	1.0000	0.9593	1.0000	1.0000	0.5168	1.0000	0.0003
29	1.0000	1.0000	0.4549	0.9981	0.0055	0.4943	1.0000	0.0035	1.0000
30	1.0000	1.0000	0.9996	1.0000	0.3752	0.9998	1.0000	0.3016	0.8822
31	0.9998	0.9999	1.0000	1.0000	0.9648	1.0000	1.0000	0.9376	0.2182
32	0.3392	0.3824	1.0000	0.9887	1.0000	1.0000	0.6769	1.0000	0.0007
33	0.9276	0.9464	1.0000	1.0000	1.0000	1.0000	0.9957	0.9999	0.0237
34	1.0000	1.0000	1.0000	1.0000	0.6418	1.0000	1.0000	0.5558	0.6628
35	1.0000	1.0000	0.9928	1.0000	0.1953	0.9952	1.0000	0.1481	0.9731
36	0.2627	0.3003	1.0000	0.9748	1.0000	1.0000	0.5823	1.0000	0.0004

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_dff_trans

i/j	10	11	12	13	14	15	16	17	18
1	1.0000	1.0000	1.0000	0.9752	1.0000	1.0000	<.0001	0.0974	0.9974
2	1.0000	1.0000	1.0000	0.9643	1.0000	1.0000	0.0001	0.1159	0.9985
3	1.0000	1.0000	0.9998	0.0050	0.9679	1.0000	0.4423	1.0000	1.0000
4	1.0000	1.0000	1.0000	0.2644	1.0000	1.0000	0.0134	0.8424	1.0000
5	0.7031	0.6757	0.4141	<.0001	0.1093	0.8597	0.9999	1.0000	0.9937
6	1.0000	1.0000	0.9999	0.0061	0.9764	1.0000	0.4043	1.0000	1.0000
7	1.0000	1.0000	1.0000	0.8130	1.0000	1.0000	0.0006	0.2952	1.0000
8	0.6196	0.5907	0.3367	<.0001	0.0798	0.7970	1.0000	1.0000	0.9858
9	0.5999	0.6286	0.8557	1.0000	0.9943	0.4085	<.0001	<.0001	0.1133
10		1.0000	1.0000	0.3071	1.0000	1.0000	0.0104	0.8015	1.0000
11	1.0000		1.0000	0.3311	1.0000	1.0000	0.0090	0.7780	1.0000
12	1.0000	1.0000		0.5842	1.0000	1.0000	0.0024	0.5249	1.0000
13	0.3071	0.3311	0.5842		0.9326	0.1742	<.0001	<.0001	0.0340
14	1.0000	1.0000	1.0000	0.9326		1.0000	0.0002	0.1609	0.9996
15	1.0000	1.0000	1.0000	0.1742	1.0000		0.0252	0.9221	1.0000
16	0.0104	0.0090	0.0024	<.0001	0.0002	0.0252		0.9992	0.1385
17	0.8015	0.7780	0.5249	<.0001	0.1609	0.9221	0.9992		0.9982
18	1.0000	1.0000	1.0000	0.0340	0.9996	1.0000	0.1385	0.9982	
19	1.0000	1.0000	1.0000	0.5120	1.0000	1.0000	0.0035	0.5971	1.0000
20	0.6334	0.6047	0.3486	<.0001	0.0841	0.8080	1.0000	1.0000	0.9875
21	1.0000	1.0000	1.0000	0.6285	1.0000	1.0000	0.0019	0.4808	1.0000
22	1.0000	1.0000	0.9995	0.0037	0.9526	1.0000	0.4969	1.0000	1.0000
23	0.9997	0.9996	0.9909	0.0010	0.8227	1.0000	0.7379	1.0000	1.0000
24	0.2924	0.2701	0.1172	<.0001	0.0189	0.4659	1.0000	1.0000	0.8621
25	0.9997	0.9998	1.0000	0.9999	1.0000	0.9965	<.0001	0.0143	0.8937
26	0.8693	0.8866	0.9797	1.0000	0.9999	0.7170	<.0001	0.0004	0.3014
27	1.0000	1.0000	1.0000	0.3689	1.0000	1.0000	0.0073	0.7406	1.0000
28	0.9416	0.9303	0.7583	<.0001	0.3263	0.9863	0.9888	1.0000	0.9999
29	0.9990	0.9993	1.0000	1.0000	1.0000	0.9915	<.0001	0.0095	0.8390
30	1.0000	1.0000	1.0000	0.6264	1.0000	1.0000	0.0019	0.4829	1.0000
31	1.0000	1.0000	1.0000	0.0761	1.0000	1.0000	0.0669	0.9857	1.0000
32	0.9818	0.9770	0.8774	0.0001	0.4744	0.9973	0.9594	1.0000	1.0000
33	1.0000	1.0000	0.9998	0.0055	0.9728	1.0000	0.4211	1.0000	1.0000
34	1.0000	1.0000	1.0000	0.3612	1.0000	1.0000	0.0076	0.7483	1.0000
35	1.0000	1.0000	1.0000	0.8348	1.0000	1.0000	0.0005	0.2725	1.0000
36	0.9621	0.9538	0.8116	<.0001	0.3834	0.9925	0.9803	1.0000	1.0000

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_dff_trans

i/j	19	20	21	22	23	24	25	26	27
1	1.0000	0.0476	1.0000	0.8889	0.6987	0.0096	1.0000	1.0000	1.0000
2	1.0000	0.0579	1.0000	0.9144	0.7433	0.0121	1.0000	1.0000	1.0000
3	0.9999	0.9999	0.9996	1.0000	1.0000	0.9924	0.5411	0.0786	1.0000
4	1.0000	0.6868	1.0000	1.0000	0.9999	0.3376	0.9994	0.8321	1.0000
5	0.4835	1.0000	0.3733	1.0000	1.0000	1.0000	0.0084	0.0002	0.6333
6	1.0000	0.9999	0.9998	1.0000	1.0000	0.9889	0.5815	0.0913	1.0000
7	1.0000	0.1706	1.0000	0.9907	0.9376	0.0453	1.0000	0.9984	1.0000
8	0.4008	1.0000	0.2999	1.0000	1.0000	1.0000	0.0055	0.0001	0.5471
9	0.8036	<.0001	0.8834	0.0167	0.0051	<.0001	1.0000	1.0000	0.6711
10	1.0000	0.6334	1.0000	1.0000	0.9997	0.2924	0.9997	0.8693	1.0000
11	1.0000	0.6047	1.0000	1.0000	0.9996	0.2701	0.9998	0.8866	1.0000
12	1.0000	0.3486	1.0000	0.9995	0.9909	0.1172	1.0000	0.9797	1.0000
13	0.5120	<.0001	0.6285	0.0037	0.0010	<.0001	0.9999	1.0000	0.3689
14	1.0000	0.0841	1.0000	0.9526	0.8227	0.0189	1.0000	0.9999	1.0000
15	1.0000	0.8080	1.0000	1.0000	1.0000	0.4659	0.9965	0.7170	1.0000
16	0.0035	1.0000	0.0019	0.4969	0.7379	1.0000	<.0001	<.0001	0.0073
17	0.5971	1.0000	0.4808	1.0000	1.0000	1.0000	0.0143	0.0004	0.7406
18	1.0000	0.9875	1.0000	1.0000	1.0000	0.8621	0.8937	0.3014	1.0000
19		0.4137	1.0000	0.9998	0.9956	0.1497	1.0000	0.9649	1.0000
20	0.4137		0.3112	1.0000	1.0000	1.0000	0.0059	0.0001	0.5611
21	1.0000	0.3112		0.9991	0.9864	0.1001	1.0000	0.9861	1.0000
22	0.9998	1.0000	0.9991		1.0000	0.9957	0.4854	0.0635	1.0000
23	0.9956	1.0000	0.9864	1.0000		0.9998	0.2671	0.0227	0.9993
24	0.1497	1.0000	0.1001	0.9957	0.9998		0.0009	<.0001	0.2387
25	1.0000	0.0059	1.0000	0.4854	0.2671	0.0009		1.0000	0.9999
26	0.9649	0.0001	0.9861	0.0635	0.0227	<.0001	1.0000		0.9098
27	1.0000	0.5611	1.0000	1.0000	0.9993	0.2387	0.9999	0.9098	
28	0.8167	1.0000	0.7187	1.0000	1.0000	1.0000	0.0413	0.0017	0.9106
29	1.0000	0.0038	1.0000	0.4018	0.2073	0.0005	1.0000	1.0000	0.9996
30	1.0000	0.3129	1.0000	0.9991	0.9867	0.1008	1.0000	0.9858	1.0000
31	1.0000	0.9428	1.0000	1.0000	1.0000	0.7001	0.9697	0.4837	1.0000
32	0.9160	1.0000	0.8489	1.0000	1.0000	1.0000	0.0781	0.0039	0.9680
33	1.0000	0.9999	0.9997	1.0000	1.0000	0.9906	0.5634	0.0854	1.0000
34	1.0000	0.5698	1.0000	1.0000	0.9994	0.2448	0.9999	0.9054	1.0000
35	1.0000	0.1551	1.0000	0.9878	0.9257	0.0402	1.0000	0.9989	1.0000
36	0.8625	1.0000	0.7760	1.0000	1.0000	1.0000	0.0539	0.0023	0.9389

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_dff_trans

i/j	28	29	30	31	32	33	34	35	36
1	0.2173	1.0000	1.0000	0.9998	0.3392	0.9276	1.0000	1.0000	0.2627
2	0.2508	1.0000	1.0000	0.9999	0.3824	0.9464	1.0000	1.0000	0.3003
3	1.0000	0.4549	0.9996	1.0000	1.0000	1.0000	1.0000	0.9928	1.0000
4	0.9593	0.9981	1.0000	1.0000	0.9887	1.0000	1.0000	1.0000	0.9748
5	1.0000	0.0055	0.3752	0.9648	1.0000	1.0000	0.6418	0.1953	1.0000
6	1.0000	0.4943	0.9998	1.0000	1.0000	1.0000	1.0000	0.9952	1.0000
7	0.5168	1.0000	1.0000	1.0000	0.6769	0.9957	1.0000	1.0000	0.5823
8	1.0000	0.0035	0.3016	0.9376	1.0000	0.9999	0.5558	0.1481	1.0000
9	0.0003	1.0000	0.8822	0.2182	0.0007	0.0237	0.6628	0.9731	0.0004
10	0.9416	0.9990	1.0000	1.0000	0.9818	1.0000	1.0000	1.0000	0.9621
11	0.9303	0.9993	1.0000	1.0000	0.9770	1.0000	1.0000	1.0000	0.9538
12	0.7583	1.0000	1.0000	1.0000	0.8774	0.9998	1.0000	1.0000	0.8116
13	<.0001	1.0000	0.6264	0.0761	0.0001	0.0055	0.3612	0.8348	<.0001
14	0.3263	1.0000	1.0000	1.0000	0.4744	0.9728	1.0000	1.0000	0.3834
15	0.9863	0.9915	1.0000	1.0000	0.9973	1.0000	1.0000	1.0000	0.9925
16	0.9888	<.0001	0.0019	0.0669	0.9594	0.4211	0.0076	0.0005	0.9803
17	1.0000	0.0095	0.4829	0.9857	1.0000	1.0000	0.7483	0.2725	1.0000
18	0.9999	0.8390	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
19	0.8167	1.0000	1.0000	1.0000	0.9160	1.0000	1.0000	1.0000	0.8625
20	1.0000	0.0038	0.3129	0.9428	1.0000	0.9999	0.5698	0.1551	1.0000
21	0.7187	1.0000	1.0000	1.0000	0.8489	0.9997	1.0000	1.0000	0.7760
22	1.0000	0.4018	0.9991	1.0000	1.0000	1.0000	1.0000	0.9878	1.0000
23	1.0000	0.2073	0.9867	1.0000	1.0000	1.0000	0.9994	0.9257	1.0000
24	1.0000	0.0005	0.1008	0.7001	1.0000	0.9906	0.2448	0.0402	1.0000
25	0.0413	1.0000	1.0000	0.9697	0.0781	0.5634	0.9999	1.0000	0.0539
26	0.0017	1.0000	0.9858	0.4837	0.0039	0.0854	0.9054	0.9989	0.0023
27	0.9106	0.9996	1.0000	1.0000	0.9680	1.0000	1.0000	1.0000	0.9389
28		0.0286	0.7207	0.9989	1.0000	1.0000	0.9148	0.4876	1.0000
29	0.0286		1.0000	0.9449	0.0558	0.4766	0.9996	1.0000	0.0378
30	0.7207	1.0000		1.0000	0.8504	0.9997	1.0000	1.0000	0.7778
31	0.9989	0.9449	1.0000		0.9999	1.0000	1.0000	1.0000	0.9996
32	1.0000	0.0558	0.8504	0.9999		1.0000	0.9700	0.6484	1.0000
33	1.0000	0.4766	0.9997	1.0000	1.0000		1.0000	0.9942	1.0000
34	0.9148	0.9996	1.0000	1.0000	0.9700	1.0000		1.0000	0.9421
35	0.4876	1.0000	1.0000	1.0000	0.6484	0.9942	1.0000		0.5529
36	1.0000	0.0378	0.7778	0.9996	1.0000	1.0000	0.9421	0.5529	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

proteina	tempo	av_thick_ trans LSMEAN	LSMEAN Number
albumina	24h	4.22425265	1
albumina	48h	4.20738705	2
albumina	72h	3.82439367	3
albumina	ades	4.01926564	4
c3b	24h	3.60309690	5
c3b	48h	3.85086451	6
c3b	72h	4.14077773	7
c3b	ades	3.59341727	8
fibrino	24h	4.53416373	9
fibrino	48h	4.03013492	10
fibrino	72h	4.03888996	11
fibrino	ades	4.09558933	12
his	24h	4.60180270	13
his	48h	4.19104239	14
his	72h	3.99181875	15
his	ades	3.35828834	16
iga	24h	3.63285424	17
iga	48h	3.94345525	18
iga	72h	4.07168232	19
iga	ades	3.58384002	20
lacto	24h	3.98087312	21
lacto	48h	3.81848841	22
lacto	72h	3.76859316	23
lacto	ades	3.51598052	24
muc_1	24h	4.31423214	25
muc_1	48h	4.48078088	26
muc_1	72h	4.04381756	27
muc_1	ades	3.66464078	28
muc_2	24h	4.32972222	29
muc_2	48h	4.10064410	30
muc_2	72h	3.94317226	31
muc_2	ades	3.70587595	32
saliva	24h	3.86091127	33
saliva	48h	4.05380471	34
saliva	72h	4.14418089	35
saliva	ades	3.70953408	36

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_thick_trans

i/j	1	2	3	4	5	6	7	8	9
1		1.0000	0.8611	1.0000	0.0542	0.9323	1.0000	0.0444	0.9951
2	1.0000		0.9103	1.0000	0.0757	0.9613	1.0000	0.0626	0.9885
3	0.8611	0.9103		1.0000	1.0000	1.0000	0.9931	1.0000	0.0072
4	1.0000	1.0000	1.0000		0.8011	1.0000	1.0000	0.7602	0.3273
5	0.0542	0.0757	1.0000	0.8011		0.9999	0.2379	1.0000	<.0001
6	0.9323	0.9613	1.0000	1.0000	0.9999		0.9985	0.9999	0.0138
7	1.0000	1.0000	0.9931	1.0000	0.2379	0.9985		0.2053	0.8816
8	0.0444	0.0626	1.0000	0.7602	1.0000	0.9999	0.2053		<.0001
9	0.9951	0.9885	0.0072	0.3273	<.0001	0.0138	0.8816	<.0001	
10	1.0000	1.0000	1.0000	1.0000	0.7550	1.0000	1.0000	0.7105	0.3757
11	1.0000	1.0000	1.0000	1.0000	0.7149	1.0000	1.0000	0.6682	0.4169
12	1.0000	1.0000	0.9996	1.0000	0.4304	1.0000	1.0000	0.3843	0.7017
13	0.9233	0.8784	0.0012	0.1137	<.0001	0.0025	0.5897	<.0001	1.0000
14	1.0000	1.0000	0.9453	1.0000	0.1031	0.9794	1.0000	0.0860	0.9768
15	1.0000	1.0000	1.0000	1.0000	0.8951	1.0000	1.0000	0.8659	0.2218
16	<.0001	0.0002	0.5636	0.0230	0.9999	0.4300	0.0011	1.0000	<.0001
17	0.0967	0.1310	1.0000	0.9014	1.0000	1.0000	0.3580	1.0000	<.0001
18	0.9991	0.9997	1.0000	1.0000	0.9792	1.0000	1.0000	0.9695	0.0980
19	1.0000	1.0000	0.9999	1.0000	0.5509	1.0000	1.0000	0.5014	0.5822
20	0.0362	0.0516	1.0000	0.7166	1.0000	0.9997	0.1763	1.0000	<.0001
21	1.0000	1.0000	1.0000	1.0000	0.9228	1.0000	1.0000	0.8986	0.1870
22	0.8407	0.8946	1.0000	1.0000	1.0000	1.0000	0.9907	1.0000	0.0062
23	0.6171	0.7006	1.0000	0.9999	1.0000	1.0000	0.9348	1.0000	0.0017
24	0.0075	0.0113	0.9954	0.3791	1.0000	0.9835	0.0503	1.0000	<.0001
25	1.0000	1.0000	0.4434	0.9979	0.0070	0.5777	1.0000	0.0055	1.0000
26	0.9999	0.9995	0.0255	0.5872	<.0001	0.0452	0.9795	<.0001	1.0000
27	1.0000	1.0000	1.0000	1.0000	0.6914	1.0000	1.0000	0.6436	0.4409
28	0.1688	0.2205	1.0000	0.9639	1.0000	1.0000	0.5122	1.0000	<.0001
29	1.0000	1.0000	0.3697	0.9949	0.0047	0.4984	1.0000	0.0037	1.0000
30	1.0000	1.0000	0.9994	1.0000	0.4061	0.9999	1.0000	0.3611	0.7255
31	0.9991	0.9997	1.0000	1.0000	0.9795	1.0000	1.0000	0.9699	0.0974
32	0.3126	0.3874	1.0000	0.9941	1.0000	1.0000	0.7191	1.0000	0.0003
33	0.9510	0.9734	1.0000	1.0000	0.9998	1.0000	0.9992	0.9997	0.0174
34	1.0000	1.0000	1.0000	1.0000	0.6421	1.0000	1.0000	0.5929	0.4907
35	1.0000	1.0000	0.9918	1.0000	0.2261	0.9981	1.0000	0.1947	0.8915
36	0.3281	0.4046	1.0000	0.9951	1.0000	1.0000	0.7360	1.0000	0.0003

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_thick_trans

i/j	10	11	12	13	14	15	16	17	18
1	1.0000	1.0000	1.0000	0.9233	1.0000	1.0000	<.0001	0.0967	0.9991
2	1.0000	1.0000	1.0000	0.8784	1.0000	1.0000	0.0002	0.1310	0.9997
3	1.0000	1.0000	0.9996	0.0012	0.9453	1.0000	0.5636	1.0000	1.0000
4	1.0000	1.0000	1.0000	0.1137	1.0000	1.0000	0.0230	0.9014	1.0000
5	0.7550	0.7149	0.4304	<.0001	0.1031	0.8951	0.9999	1.0000	0.9792
6	1.0000	1.0000	1.0000	0.0025	0.9794	1.0000	0.4300	1.0000	1.0000
7	1.0000	1.0000	1.0000	0.5897	1.0000	1.0000	0.0011	0.3580	1.0000
8	0.7105	0.6682	0.3843	<.0001	0.0860	0.8659	1.0000	1.0000	0.9695
9	0.3757	0.4169	0.7017	1.0000	0.9768	0.2218	<.0001	<.0001	0.0980
10		1.0000	1.0000	0.1377	1.0000	1.0000	0.0180	0.8695	1.0000
11	1.0000		1.0000	0.1598	1.0000	1.0000	0.0147	0.8398	1.0000
12	1.0000	1.0000		0.3657	1.0000	1.0000	0.0036	0.5809	1.0000
13	0.1377	0.1598	0.3657		0.8223	0.0678	<.0001	<.0001	0.0244
14	1.0000	1.0000	1.0000	0.8223		1.0000	0.0002	0.1728	0.9999
15	1.0000	1.0000	1.0000	0.0678	1.0000		0.0419	0.9578	1.0000
16	0.0180	0.0147	0.0036	<.0001	0.0002	0.0419		0.9994	0.1084
17	0.8695	0.8398	0.5809	<.0001	0.1728	0.9578	0.9994		0.9949
18	1.0000	1.0000	1.0000	0.0244	0.9999	1.0000	0.1084	0.9949	
19	1.0000	1.0000	1.0000	0.2656	1.0000	1.0000	0.0066	0.7005	1.0000
20	0.6641	0.6202	0.3410	<.0001	0.0716	0.8327	1.0000	1.0000	0.9569
21	1.0000	1.0000	1.0000	0.0544	1.0000	1.0000	0.0526	0.9718	1.0000
22	1.0000	1.0000	0.9993	0.0010	0.9340	1.0000	0.5939	1.0000	1.0000
23	0.9998	0.9996	0.9884	0.0002	0.7750	1.0000	0.8240	1.0000	1.0000
24	0.3305	0.2939	0.1198	<.0001	0.0167	0.5137	1.0000	1.0000	0.7530
25	0.9989	0.9994	1.0000	0.9987	1.0000	0.9907	<.0001	0.0144	0.9376
26	0.6424	0.6857	0.9046	1.0000	0.9985	0.4477	<.0001	0.0002	0.2392
27	1.0000	1.0000	1.0000	0.1733	1.0000	1.0000	0.0130	0.8215	1.0000
28	0.9474	0.9305	0.7374	<.0001	0.2800	0.9883	0.9959	1.0000	0.9993
29	0.9972	0.9984	1.0000	0.9995	1.0000	0.9813	<.0001	0.0099	0.9018
30	1.0000	1.0000	1.0000	0.3890	1.0000	1.0000	0.0031	0.5550	1.0000
31	1.0000	1.0000	1.0000	0.0243	0.9999	1.0000	0.1090	0.9949	1.0000
32	0.9898	0.9848	0.8923	<.0001	0.4666	0.9988	0.9723	1.0000	1.0000
33	1.0000	1.0000	1.0000	0.0033	0.9866	1.0000	0.3822	1.0000	1.0000
34	1.0000	1.0000	1.0000	0.2033	1.0000	1.0000	0.0103	0.7813	1.0000
35	1.0000	1.0000	1.0000	0.6071	1.0000	1.0000	0.0010	0.3428	1.0000
36	0.9915	0.9871	0.9024	<.0001	0.4849	0.9991	0.9681	1.0000	1.0000

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_thick_trans

i/j	19	20	21	22	23	24	25	26	27
1	1.0000	0.0362	1.0000	0.8407	0.6171	0.0075	1.0000	0.9999	1.0000
2	1.0000	0.0516	1.0000	0.8946	0.7006	0.0113	1.0000	0.9995	1.0000
3	0.9999	1.0000	1.0000	1.0000	1.0000	0.9954	0.4434	0.0255	1.0000
4	1.0000	0.7166	1.0000	1.0000	0.9999	0.3791	0.9979	0.5872	1.0000
5	0.5509	1.0000	0.9228	1.0000	1.0000	1.0000	0.0070	<.0001	0.6914
6	1.0000	0.9997	1.0000	1.0000	1.0000	0.9835	0.5777	0.0452	1.0000
7	1.0000	0.1763	1.0000	0.9907	0.9348	0.0503	1.0000	0.9795	1.0000
8	0.5014	1.0000	0.8986	1.0000	1.0000	1.0000	0.0055	<.0001	0.6436
9	0.5822	<.0001	0.1870	0.0062	0.0017	<.0001	1.0000	1.0000	0.4409
10	1.0000	0.6641	1.0000	1.0000	0.9998	0.3305	0.9989	0.6424	1.0000
11	1.0000	0.6202	1.0000	1.0000	0.9996	0.2939	0.9994	0.6857	1.0000
12	1.0000	0.3410	1.0000	0.9993	0.9884	0.1198	1.0000	0.9046	1.0000
13	0.2656	<.0001	0.0544	0.0010	0.0002	<.0001	0.9987	1.0000	0.1733
14	1.0000	0.0716	1.0000	0.9340	0.7750	0.0167	1.0000	0.9985	1.0000
15	1.0000	0.8327	1.0000	1.0000	1.0000	0.5137	0.9907	0.4477	1.0000
16	0.0066	1.0000	0.0526	0.5939	0.8240	1.0000	<.0001	<.0001	0.0130
17	0.7005	1.0000	0.9718	1.0000	1.0000	1.0000	0.0144	0.0002	0.8215
18	1.0000	0.9569	1.0000	1.0000	1.0000	0.7530	0.9376	0.2392	1.0000
19		0.4533	1.0000	0.9999	0.9966	0.1799	1.0000	0.8285	1.0000
20	0.4533		0.8703	1.0000	1.0000	1.0000	0.0043	<.0001	0.5950
21	1.0000	0.8703		1.0000	1.0000	0.5698	0.9846	0.3949	1.0000
22	0.9999	1.0000	1.0000		1.0000	0.9967	0.4147	0.0224	1.0000
23	0.9966	1.0000	1.0000	1.0000		0.9999	0.2109	0.0068	0.9994
24	0.1799	1.0000	0.5698	0.9967	0.9999		0.0007	<.0001	0.2744
25	1.0000	0.0043	0.9846	0.4147	0.2109	0.0007		1.0000	0.9996
26	0.8285	<.0001	0.3949	0.0224	0.0068	<.0001	1.0000		0.7094
27	1.0000	0.5950	1.0000	1.0000	0.9994	0.2744	0.9996	0.7094	
28	0.8361	1.0000	0.9931	1.0000	1.0000	1.0000	0.0297	0.0004	0.9195
29	0.9998	0.0029	0.9709	0.3432	0.1646	0.0004	1.0000	1.0000	0.9988
30	1.0000	0.3192	1.0000	0.9991	0.9854	0.1094	1.0000	0.9173	1.0000
31	1.0000	0.9573	1.0000	1.0000	1.0000	0.7543	0.9370	0.2382	1.0000
32	0.9468	1.0000	0.9994	1.0000	1.0000	1.0000	0.0700	0.0013	0.9812
33	1.0000	0.9993	1.0000	1.0000	1.0000	0.9750	0.6289	0.0556	1.0000
34	1.0000	0.5438	1.0000	1.0000	0.9989	0.2374	0.9998	0.7552	1.0000
35	1.0000	0.1668	1.0000	0.9891	0.9276	0.0469	1.0000	0.9823	1.0000
36	0.9529	1.0000	0.9996	1.0000	1.0000	1.0000	0.0751	0.0014	0.9839

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: av_thick_trans

i/j	28	29	30	31	32	33	34	35	36
1	0.1688	1.0000	1.0000	0.9991	0.3126	0.9510	1.0000	1.0000	0.3281
2	0.2205	1.0000	1.0000	0.9997	0.3874	0.9734	1.0000	1.0000	0.4046
3	1.0000	0.3697	0.9994	1.0000	1.0000	1.0000	1.0000	0.9918	1.0000
4	0.9639	0.9949	1.0000	1.0000	0.9941	1.0000	1.0000	1.0000	0.9951
5	1.0000	0.0047	0.4061	0.9795	1.0000	0.9998	0.6421	0.2261	1.0000
6	1.0000	0.4984	0.9999	1.0000	1.0000	1.0000	1.0000	0.9981	1.0000
7	0.5122	1.0000	1.0000	1.0000	0.7191	0.9992	1.0000	1.0000	0.7360
8	1.0000	0.0037	0.3611	0.9699	1.0000	0.9997	0.5929	0.1947	1.0000
9	<.0001	1.0000	0.7255	0.0974	0.0003	0.0174	0.4907	0.8915	0.0003
10	0.9474	0.9972	1.0000	1.0000	0.9898	1.0000	1.0000	1.0000	0.9915
11	0.9305	0.9984	1.0000	1.0000	0.9848	1.0000	1.0000	1.0000	0.9871
12	0.7374	1.0000	1.0000	1.0000	0.8923	1.0000	1.0000	1.0000	0.9024
13	<.0001	0.9995	0.3890	0.0243	<.0001	0.0033	0.2033	0.6071	<.0001
14	0.2800	1.0000	1.0000	0.9999	0.4666	0.9866	1.0000	1.0000	0.4849
15	0.9883	0.9813	1.0000	1.0000	0.9988	1.0000	1.0000	1.0000	0.9991
16	0.9959	<.0001	0.0031	0.1090	0.9723	0.3822	0.0103	0.0010	0.9681
17	1.0000	0.0099	0.5550	0.9949	1.0000	1.0000	0.7813	0.3428	1.0000
18	0.9993	0.9018	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
19	0.8361	0.9998	1.0000	1.0000	0.9468	1.0000	1.0000	1.0000	0.9529
20	1.0000	0.0029	0.3192	0.9573	1.0000	0.9993	0.5438	0.1668	1.0000
21	0.9931	0.9709	1.0000	1.0000	0.9994	1.0000	1.0000	1.0000	0.9996
22	1.0000	0.3432	0.9991	1.0000	1.0000	1.0000	1.0000	0.9891	1.0000
23	1.0000	0.1646	0.9854	1.0000	1.0000	1.0000	0.9989	0.9276	1.0000
24	1.0000	0.0004	0.1094	0.7543	1.0000	0.9750	0.2374	0.0469	1.0000
25	0.0297	1.0000	1.0000	0.9370	0.0700	0.6289	0.9998	1.0000	0.0751
26	0.0004	1.0000	0.9173	0.2382	0.0013	0.0556	0.7552	0.9823	0.0014
27	0.9195	0.9988	1.0000	1.0000	0.9812	1.0000	1.0000	1.0000	0.9839
28		0.0210	0.7139	0.9993	1.0000	1.0000	0.8939	0.4949	1.0000
29	0.0210		1.0000	0.9010	0.0513	0.5497	0.9994	1.0000	0.0553
30	0.7139	1.0000		1.0000	0.8774	1.0000	1.0000	1.0000	0.8883
31	0.9993	0.9010	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000
32	1.0000	0.0513	0.8774	1.0000		1.0000	0.9719	0.7030	1.0000
33	1.0000	0.5497	1.0000	1.0000	1.0000		1.0000	0.9990	1.0000
34	0.8939	0.9994	1.0000	1.0000	0.9719	1.0000		1.0000	0.9757
35	0.4949	1.0000	1.0000	1.0000	0.7030	0.9990	1.0000		0.7203
36	1.0000	0.0553	0.8883	1.0000	1.0000	1.0000	0.9757	0.7203	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

proteina	tempo	biom_trans LSMEAN	LSMEAN Number
albumina	24h	1.27450730	1
albumina	48h	1.26985232	2
albumina	72h	1.19210839	3
albumina	ades	1.23328832	4
c3b	24h	1.14247522	5
c3b	48h	1.19321539	6
c3b	72h	1.25763957	7
c3b	ades	1.13871174	8
fibrino	24h	1.33094097	9
fibrino	48h	1.23529857	10
fibrino	72h	1.23640220	11
fibrino	ades	1.24892185	12
his	24h	1.34354572	13
his	48h	1.26577082	14
his	72h	1.22729836	15
his	ades	1.08835463	16
iga	24h	1.19225753	17
iga	48h	1.23759307	18
iga	72h	1.25890600	19
iga	ades	1.17065065	20
lacto	24h	1.14693395	21
lacto	48h	1.20796970	22
lacto	72h	1.24451878	23
lacto	ades	1.13975049	24
muc_1	24h	1.24901403	25
muc_1	48h	1.18960528	26
muc_1	72h	1.17948772	27
muc_1	ades	1.12477767	28
muc_2	24h	1.29077643	29
muc_2	48h	1.30838532	30
muc_2	72h	1.23829396	31
muc_2	ades	1.15744012	32
saliva	24h	1.29438849	33
saliva	48h	1.24907825	34
saliva	72h	1.21758182	35
saliva	ades	1.16402592	36

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: biom_trans

i/j	1	2	3	4	5	6	7	8	9
1		1.0000	0.8831	1.0000	0.0456	0.8982	1.0000	0.0311	0.9996
2	1.0000		0.9378	1.0000	0.0714	0.9475	1.0000	0.0498	0.9983
3	0.8831	0.9378		1.0000	1.0000	1.0000	0.9943	0.9999	0.0225
4	1.0000	1.0000	1.0000		0.7280	1.0000	1.0000	0.6409	0.5662
5	0.0456	0.0714	1.0000	0.7280		1.0000	0.1998	1.0000	<.0001
6	0.8982	0.9475	1.0000	1.0000	1.0000		0.9957	0.9998	0.0254
7	1.0000	1.0000	0.9943	1.0000	0.1998	0.9957		0.1491	0.9702
8	0.0311	0.0498	0.9999	0.6409	1.0000	0.9998	0.1491		<.0001
9	0.9996	0.9983	0.0225	0.5662	<.0001	0.0254	0.9702	<.0001	
10	1.0000	1.0000	1.0000	1.0000	0.6824	1.0000	1.0000	0.5923	0.6152
11	1.0000	1.0000	1.0000	1.0000	0.6564	1.0000	1.0000	0.5653	0.6418
12	1.0000	1.0000	0.9996	1.0000	0.3597	0.9997	1.0000	0.2837	0.8884
13	0.9872	0.9680	0.0053	0.2828	<.0001	0.0061	0.8267	<.0001	1.0000
14	1.0000	1.0000	0.9682	1.0000	0.1032	0.9740	1.0000	0.0735	0.9948
15	1.0000	1.0000	1.0000	1.0000	0.8455	1.0000	1.0000	0.7753	0.4223
16	<.0001	<.0001	0.4197	0.0114	0.9998	0.3946	0.0005	1.0000	<.0001
17	0.8852	0.9392	1.0000	1.0000	1.0000	1.0000	0.9946	0.9999	0.0229
18	1.0000	1.0000	1.0000	1.0000	0.6279	1.0000	1.0000	0.5361	0.6701
19	1.0000	1.0000	0.9923	1.0000	0.1815	0.9941	1.0000	0.1344	0.9764
20	0.4174	0.5283	1.0000	0.9974	1.0000	1.0000	0.8067	1.0000	0.0017
21	0.0701	0.1067	1.0000	0.8186	1.0000	1.0000	0.2744	1.0000	<.0001
22	0.9928	0.9979	1.0000	1.0000	0.9944	1.0000	1.0000	0.9866	0.1062
23	1.0000	1.0000	0.9999	1.0000	0.4597	1.0000	1.0000	0.3736	0.8173
24	0.0346	0.0551	0.9999	0.6656	1.0000	0.9999	0.1620	1.0000	<.0001
25	1.0000	1.0000	0.9996	1.0000	0.3578	0.9997	1.0000	0.2819	0.8897
26	0.8441	0.9112	1.0000	1.0000	1.0000	1.0000	0.9898	1.0000	0.0172
27	0.6303	0.7378	1.0000	0.9999	1.0000	1.0000	0.9339	1.0000	0.0053
28	0.0065	0.0113	0.9913	0.3168	1.0000	0.9888	0.0420	1.0000	<.0001
29	1.0000	1.0000	0.5413	0.9995	0.0077	0.5684	1.0000	0.0049	1.0000
30	1.0000	1.0000	0.1837	0.9592	0.0008	0.1998	1.0000	0.0005	1.0000
31	1.0000	1.0000	1.0000	1.0000	0.6110	1.0000	1.0000	0.5190	0.6864
32	0.1728	0.2440	1.0000	0.9537	1.0000	1.0000	0.5040	1.0000	0.0003
33	1.0000	1.0000	0.4541	0.9983	0.0050	0.4805	1.0000	0.0031	1.0000
34	1.0000	1.0000	0.9996	1.0000	0.3564	0.9997	1.0000	0.2807	0.8906
35	0.9996	0.9999	1.0000	1.0000	0.9591	1.0000	1.0000	0.9266	0.2281
36	0.2786	0.3732	1.0000	0.9866	1.0000	1.0000	0.6638	1.0000	0.0007

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: biom_trans

i/j	10	11	12	13	14	15	16	17	18
1	1.0000	1.0000	1.0000	0.9872	1.0000	1.0000	<.0001	0.8852	1.0000
2	1.0000	1.0000	1.0000	0.9680	1.0000	1.0000	<.0001	0.9392	1.0000
3	1.0000	1.0000	0.9996	0.0053	0.9682	1.0000	0.4197	1.0000	1.0000
4	1.0000	1.0000	1.0000	0.2828	1.0000	1.0000	0.0114	1.0000	1.0000
5	0.6824	0.6564	0.3597	<.0001	0.1032	0.8455	0.9998	1.0000	0.6279
6	1.0000	1.0000	0.9997	0.0061	0.9740	1.0000	0.3946	1.0000	1.0000
7	1.0000	1.0000	1.0000	0.8267	1.0000	1.0000	0.0005	0.9946	1.0000
8	0.5923	0.5653	0.2837	<.0001	0.0735	0.7753	1.0000	0.9999	0.5361
9	0.6152	0.6418	0.8884	1.0000	0.9948	0.4223	<.0001	0.0229	0.6701
10		1.0000	1.0000	0.3221	1.0000	1.0000	0.0091	1.0000	1.0000
11	1.0000		1.0000	0.3449	1.0000	1.0000	0.0080	1.0000	1.0000
12	1.0000	1.0000		0.6398	1.0000	1.0000	0.0017	0.9996	1.0000
13	0.3221	0.3449	0.6398		0.9375	0.1841	<.0001	0.0054	0.3704
14	1.0000	1.0000	1.0000	0.9375		1.0000	0.0002	0.9690	1.0000
15	1.0000	1.0000	1.0000	0.1841	1.0000		0.0223	1.0000	1.0000
16	0.0091	0.0080	0.0017	<.0001	0.0002	0.0223		0.4163	0.0069
17	1.0000	1.0000	0.9996	0.0054	0.9690	1.0000	0.4163		1.0000
18	1.0000	1.0000	1.0000	0.3704	1.0000	1.0000	0.0069	1.0000	
19	1.0000	1.0000	1.0000	0.8485	1.0000	1.0000	0.0004	0.9926	1.0000
20	0.9955	0.9940	0.9327	0.0003	0.6279	0.9996	0.8846	1.0000	0.9920
21	0.7798	0.7570	0.4610	<.0001	0.1502	0.9098	0.9992	1.0000	0.7313
22	1.0000	1.0000	1.0000	0.0318	0.9994	1.0000	0.1410	1.0000	1.0000
23	1.0000	1.0000	1.0000	0.5326	1.0000	1.0000	0.0030	0.9999	1.0000
24	0.6175	0.5907	0.3036	<.0001	0.0809	0.7960	0.9999	0.9999	0.5615
25	1.0000	1.0000	1.0000	0.6420	1.0000	1.0000	0.0017	0.9996	1.0000
26	1.0000	1.0000	0.9990	0.0039	0.9513	1.0000	0.4786	1.0000	1.0000
27	0.9997	0.9996	0.9861	0.0011	0.8199	1.0000	0.7209	1.0000	0.9993
28	0.2778	0.2578	0.0958	<.0001	0.0178	0.4485	1.0000	0.9910	0.2372
29	0.9997	0.9998	1.0000	0.9999	1.0000	0.9967	<.0001	0.5450	0.9999
30	0.9713	0.9767	0.9990	1.0000	1.0000	0.9008	<.0001	0.1858	0.9815
31	1.0000	1.0000	1.0000	0.3859	1.0000	1.0000	0.0064	1.0000	1.0000
32	0.9367	0.9257	0.7131	<.0001	0.3204	0.9848	0.9871	1.0000	0.9123
33	0.9991	0.9994	1.0000	1.0000	1.0000	0.9918	<.0001	0.4577	0.9996
34	1.0000	1.0000	1.0000	0.6435	1.0000	1.0000	0.0017	0.9996	1.0000
35	1.0000	1.0000	1.0000	0.0813	1.0000	1.0000	0.0599	1.0000	1.0000
36	0.9797	0.9749	0.8442	0.0001	0.4668	0.9969	0.9551	1.0000	0.9687

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: biom_trans

i/j	19	20	21	22	23	24	25	26	27
1	1.0000	0.4174	0.0701	0.9928	1.0000	0.0346	1.0000	0.8441	0.6303
2	1.0000	0.5283	0.1067	0.9979	1.0000	0.0551	1.0000	0.9112	0.7378
3	0.9923	1.0000	1.0000	1.0000	0.9999	0.9999	0.9996	1.0000	1.0000
4	1.0000	0.9974	0.8186	1.0000	1.0000	0.6656	1.0000	1.0000	0.9999
5	0.1815	1.0000	1.0000	0.9944	0.4597	1.0000	0.3578	1.0000	1.0000
6	0.9941	1.0000	1.0000	1.0000	1.0000	0.9999	0.9997	1.0000	1.0000
7	1.0000	0.8067	0.2744	1.0000	1.0000	0.1620	1.0000	0.9898	0.9339
8	0.1344	1.0000	1.0000	0.9866	0.3736	1.0000	0.2819	1.0000	1.0000
9	0.9764	0.0017	<.0001	0.1062	0.8173	<.0001	0.8897	0.0172	0.0053
10	1.0000	0.9955	0.7798	1.0000	1.0000	0.6175	1.0000	1.0000	0.9997
11	1.0000	0.9940	0.7570	1.0000	1.0000	0.5907	1.0000	1.0000	0.9996
12	1.0000	0.9327	0.4610	1.0000	1.0000	0.3036	1.0000	0.9990	0.9861
13	0.8485	0.0003	<.0001	0.0318	0.5326	<.0001	0.6420	0.0039	0.0011
14	1.0000	0.6279	0.1502	0.9994	1.0000	0.0809	1.0000	0.9513	0.8199
15	1.0000	0.9996	0.9098	1.0000	1.0000	0.7960	1.0000	1.0000	1.0000
16	0.0004	0.8846	0.9992	0.1410	0.0030	0.9999	0.0017	0.4786	0.7209
17	0.9926	1.0000	1.0000	1.0000	0.9999	0.9999	0.9996	1.0000	1.0000
18	1.0000	0.9920	0.7313	1.0000	1.0000	0.5615	1.0000	1.0000	0.9993
19		0.7820	0.2516	1.0000	1.0000	0.1463	1.0000	0.9865	0.9207
20	0.7820		1.0000	1.0000	0.9670	1.0000	0.9318	1.0000	1.0000
21	0.2516	1.0000		0.9983	0.5679	1.0000	0.4589	1.0000	1.0000
22	1.0000	1.0000	0.9983		1.0000	0.9893	1.0000	1.0000	1.0000
23	1.0000	0.9670	0.5679	1.0000		0.3967	1.0000	0.9998	0.9950
24	0.1463	1.0000	1.0000	0.9893	0.3967		0.3018	1.0000	1.0000
25	1.0000	0.9318	0.4589	1.0000	1.0000	0.3018		0.9990	0.9858
26	0.9865	1.0000	1.0000	1.0000	0.9998	1.0000	0.9990		1.0000
27	0.9207	1.0000	1.0000	1.0000	0.9950	1.0000	0.9858	1.0000	
28	0.0369	1.0000	1.0000	0.8715	0.1395	1.0000	0.0950	0.9953	0.9998
29	1.0000	0.1352	0.0130	0.8772	1.0000	0.0056	1.0000	0.4806	0.2638
30	1.0000	0.0254	0.0015	0.4988	0.9963	0.0006	0.9990	0.1509	0.0619
31	1.0000	0.9906	0.7158	1.0000	1.0000	0.5444	1.0000	1.0000	0.9992
32	0.4735	1.0000	1.0000	1.0000	0.8050	1.0000	0.7110	1.0000	1.0000
33	1.0000	0.0993	0.0085	0.8174	1.0000	0.0036	1.0000	0.3963	0.2038
34	1.0000	0.9312	0.4573	1.0000	1.0000	0.3006	1.0000	0.9990	0.9856
35	1.0000	1.0000	0.9821	1.0000	1.0000	0.9370	1.0000	1.0000	1.0000
36	0.6337	1.0000	1.0000	1.0000	0.9083	1.0000	0.8427	1.0000	1.0000

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect proteina*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: biom_trans

i/j	28	29	30	31	32	33	34	35	36
1	0.0065	1.0000	1.0000	1.0000	0.1728	1.0000	1.0000	0.9996	0.2786
2	0.0113	1.0000	1.0000	1.0000	0.2440	1.0000	1.0000	0.9999	0.3732
3	0.9913	0.5413	0.1837	1.0000	1.0000	0.4541	0.9996	1.0000	1.0000
4	0.3168	0.9995	0.9592	1.0000	0.9537	0.9983	1.0000	1.0000	0.9866
5	1.0000	0.0077	0.0008	0.6110	1.0000	0.0050	0.3564	0.9591	1.0000
6	0.9888	0.5684	0.1998	1.0000	1.0000	0.4805	0.9997	1.0000	1.0000
7	0.0420	1.0000	1.0000	1.0000	0.5040	1.0000	1.0000	1.0000	0.6638
8	1.0000	0.0049	0.0005	0.5190	1.0000	0.0031	0.2807	0.9266	1.0000
9	<.0001	1.0000	1.0000	0.6864	0.0003	1.0000	0.8906	0.2281	0.0007
10	0.2778	0.9997	0.9713	1.0000	0.9367	0.9991	1.0000	1.0000	0.9797
11	0.2578	0.9998	0.9767	1.0000	0.9257	0.9994	1.0000	1.0000	0.9749
12	0.0958	1.0000	0.9990	1.0000	0.7131	1.0000	1.0000	1.0000	0.8442
13	<.0001	0.9999	1.0000	0.3859	<.0001	1.0000	0.6435	0.0813	0.0001
14	0.0178	1.0000	1.0000	1.0000	0.3204	1.0000	1.0000	1.0000	0.4668
15	0.4485	0.9967	0.9008	1.0000	0.9848	0.9918	1.0000	1.0000	0.9969
16	1.0000	<.0001	<.0001	0.0064	0.9871	<.0001	0.0017	0.0599	0.9551
17	0.9910	0.5450	0.1858	1.0000	1.0000	0.4577	0.9996	1.0000	1.0000
18	0.2372	0.9999	0.9815	1.0000	0.9123	0.9996	1.0000	1.0000	0.9687
19	0.0369	1.0000	1.0000	1.0000	0.4735	1.0000	1.0000	1.0000	0.6337
20	1.0000	0.1352	0.0254	0.9906	1.0000	0.0993	0.9312	1.0000	1.0000
21	1.0000	0.0130	0.0015	0.7158	1.0000	0.0085	0.4573	0.9821	1.0000
22	0.8715	0.8772	0.4988	1.0000	1.0000	0.8174	1.0000	1.0000	1.0000
23	0.1395	1.0000	0.9963	1.0000	0.8050	1.0000	1.0000	1.0000	0.9083
24	1.0000	0.0056	0.0006	0.5444	1.0000	0.0036	0.3006	0.9370	1.0000
25	0.0950	1.0000	0.9990	1.0000	0.7110	1.0000	1.0000	1.0000	0.8427
26	0.9953	0.4806	0.1509	1.0000	1.0000	0.3963	0.9990	1.0000	1.0000
27	0.9998	0.2638	0.0619	0.9992	1.0000	0.2038	0.9856	1.0000	1.0000
28		0.0008	<.0001	0.2256	1.0000	0.0005	0.0945	0.6828	1.0000
29	0.0008		1.0000	0.9999	0.0400	1.0000	1.0000	0.9707	0.0757
30	<.0001	1.0000		0.9840	0.0056	1.0000	0.9990	0.7282	0.0122
31	0.2256	0.9999	0.9840		0.9038	0.9997	1.0000	1.0000	0.9646
32	1.0000	0.0400	0.0056	0.9038		0.0276	0.7095	0.9987	1.0000
33	0.0005	1.0000	1.0000	0.9997	0.0276		1.0000	0.9461	0.0537
34	0.0945	1.0000	0.9990	1.0000	0.7095	1.0000		1.0000	0.8416
35	0.6828	0.9707	0.7282	1.0000	0.9987	0.9461	1.0000		0.9999
36	1.0000	0.0757	0.0122	0.9646	1.0000	0.0537	0.8416	0.9999	

D) XTT

The GLM Procedure

Dependent Variable: xtt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	383	0.01439151	0.00003758		
Corrected Total	418	40.57186822			

R-Square	Coeff Var	Root MSE	xtt Mean
0.999645	2.484636	0.006130	0.246712

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trat	8	7.57096609	0.94637076	25185.7	<.0001
tempo	3	21.49094047	7.16364682	190645	<.0001
trat*tempo	24	13.45059907	0.56044163	14915.0	<.0001
Model	35	40.55747670	1.15878505	30838.6	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

trat	tempo	xtt LSMEAN	LSMEAN Number
Albumina	24h	0.27915000	1
Albumina	48h	0.05140833	2
Albumina	72h	0.11692500	3
Albumina	adesao	0.86892500	4
C3b	24h	0.10865833	5
C3b	48h	0.12638333	6
C3b	72h	0.05020000	7
C3b	adesao	0.50130000	8
Fibrinog	24h	0.11545833	9
Fibrinog	48h	0.05203333	10
Fibrinog	72h	0.22160000	11
Fibrinog	adesao	0.25793333	12
Histatin	24h	0.10421667	13
Histatin	48h	0.11767500	14
Histatin	72h	0.07143333	15
Histatin	adesao	0.55004167	16
IgA	24h	0.08356667	17
IgA	48h	0.16550000	18
IgA	72h	0.21220000	19
IgA	adesao	0.91994167	20
Lactofer	24h	0.01356667	21
Lactofer	48h	0.04430833	22
Lactofer	72h	0.12838333	23
Lactofer	adesao	0.14472500	24
MucI	24h	0.04511667	25
MucI	48h	0.19104167	26
MucI	72h	0.27241667	27
MucI	adesao	0.82223333	28
MucII	24h	0.19505000	29
MucII	48h	0.09277500	30
MucII	72h	0.21632000	31
MucII	adesao	1.64891111	32
Saliva	24h	0.04590833	33
Saliva	48h	0.02435000	34
Saliva	72h	0.16824167	35
Saliva	adesao	0.13136667	36

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect trat*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: xtt

i/j	1	2	3	4	5	6	7	8	9
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001
3	<.0001	<.0001		<.0001	0.2535	0.0669	<.0001	<.0001	1.0000
4	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	0.2535	<.0001		<.0001	<.0001	<.0001	0.7028
6	<.0001	<.0001	0.0669	<.0001	<.0001		<.0001	<.0001	0.0080
7	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
9	<.0001	<.0001	1.0000	<.0001	0.7028	0.0080	<.0001	<.0001	
10	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001
11	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	0.0003	<.0001	0.9988	<.0001	<.0001	<.0001	0.0047
14	<.0001	<.0001	1.0000	<.0001	0.1148	0.1623	<.0001	<.0001	1.0000
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
22	<.0001	0.6063	<.0001	<.0001	<.0001	<.0001	0.9534	<.0001	<.0001
23	<.0001	<.0001	0.0033	<.0001	<.0001	1.0000	<.0001	<.0001	0.0002
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
25	<.0001	0.8419	<.0001	<.0001	<.0001	<.0001	0.9945	<.0001	<.0001
26	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
27	0.7231	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
28	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
31	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	0.9644	<.0001	<.0001	<.0001	<.0001	0.9998	<.0001	<.0001
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	<.0001	<.0001	0.9976	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect trat*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: xtt

i/j	10	11	12	13	14	15	16	17	18
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001	<.0001	0.0003	1.0000	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.9988	0.1148	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.1623	<.0001	<.0001	<.0001	<.0001
7	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	0.0047	1.0000	<.0001	<.0001	<.0001	<.0001
10		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	0.0010	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001
17	<.0001	<.0001	<.0001	<.0001	<.0001	0.0010	<.0001		<.0001
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
19	<.0001	0.1632	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
22	0.4027	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	0.0112	<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
25	0.6660	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
27	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
28	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	<.0001	<.0001	<.0001	0.0034	<.0001	<.0001	<.0001	0.0914	<.0001
31	<.0001	0.9968	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	0.8777	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000
36	<.0001	<.0001	<.0001	<.0001	0.0004	<.0001	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect trat*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: xtt

i/j	19	20	21	22	23	24	25	26	27
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.7231
2	<.0001	<.0001	<.0001	0.6063	<.0001	<.0001	0.8419	<.0001	<.0001
3	<.0001	<.0001	<.0001	<.0001	0.0033	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	0.9534	<.0001	<.0001	0.9945	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001
10	<.0001	<.0001	<.0001	0.4027	<.0001	<.0001	0.6660	<.0001	<.0001
11	0.1632	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	0.0112	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
22	<.0001	<.0001	<.0001		<.0001	<.0001	1.0000	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
25	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001		<.0001	<.0001
26	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
27	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
28	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9998	<.0001
30	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
31	0.9999	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	1.0000	<.0001	<.0001
34	<.0001	<.0001	0.0100	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	<.0001	1.0000	0.0007	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect trat*tempo
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: xtt

i/j	28	29	30	31	32	33	34	35	36
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001	<.0001	<.0001	<.0001	<.0001	0.9644	<.0001	<.0001	<.0001
3	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9976
7	<.0001	<.0001	<.0001	<.0001	<.0001	0.9998	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	<.0001	<.0001	<.0001	<.0001	<.0001	0.8777	<.0001	<.0001	<.0001
11	<.0001	<.0001	<.0001	0.9968	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	0.0034	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0004
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	0.0914	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001
19	<.0001	<.0001	<.0001	0.9999	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0100	<.0001	<.0001
22	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007
25	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001
26	<.0001	0.9998	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
27	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
28		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
29	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
31	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
36	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	