Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

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Cirurgiã-Dentista

EFEITO DO LAURIL SULFATO DE SÓDIO (LSS) E DOS FLAVORIZANTES, PRESENTES EM DENTIFRÍCIO, NO HÁLITO MATINAL E NA SABURRA LINGUAL DE INDIVÍDUOS PERIODONTALMENTE SAUDÁVEIS.

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do grau de Mestre em Clínica Odontológica, Área de Periodontia.

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SUMÁRIO

RESUMO

A halitose matinal está relacionada com a liberação de compostos sulfurados voláteis (CSV) produzidos em maiores concentrações durante o sono, mesmo em indivíduos saudáveis. Assim, os objetivos deste estudo foram de: (capítulo I) avaliar os efeitos do lauril sulfato de sódio (LSS) e dos flavorizantes (capítulo II), presentes em um dentifrício, na formação de CSV e da saburra lingual no hálito matinal. Os dois estudos consistiram de um delineamento cruzado, randomizado, simples-cego em uma amostra total de 75 indivíduos periodontalmente saudáveis. No capítulo I, 25 indivíduos foram divididos aleatoriamente em dois grupos experimentais: dentifrício teste, contendo LSS e dentifrício placebo; no capítulo II, 50 indivíduos foram divididos aleatoriamente em dois grupos experimentais: dentifrício teste, contendo flavorizantes e dentifrício placebo. Os voluntários receberam o dentifrício designado e uma escova dental nova para um regime de 3 escovações diárias, para 2 períodos de 30 dias. Entre os períodos, foi adotado um intervalo de 7 dias, durante o qual todos os voluntários utilizaram o dentifrício placebo. Os parâmetros avaliados foram: nível de CSV por meio do teste organoléptico (ORG) e do monitor de sulfetos, antes (H1) e após (H2) a remoção da saburra lingual; peso úmido da saburra lingual; índice de placa; índice gengival; atividade antimicrobiana dos dentifrícios pela diluição inibitória máxima e teste BANA da saburra lingual (capítulo II). As análises estatísticas dos resultados demonstraram que a presença do LSS reduziu os escores organolépticos e o H1 para o grupo teste. Quanto à presença de flavorizantes, a análise intergrupo demonstrou redução dos escores organolépticos e dos níveis de CSV, tanto para H1, como para H2, somente para o grupo teste ($p<0,05$). Teste BANA da saburra lingual não apresentou diferença entre os grupos. Dentro dos limites deste trabalho, pode-se concluir que LSS, presente em dentifrício, foi eficaz para controlar a formação de CSV no hálito matinal medido antes da remoção da saburra, sem, no entanto, reduzir a saburra lingual. Porém, quando associado aos flavorizantes, reduziu a halitose matinal detectada pela redução dos níveis de CSV e dos escores organolépticos, sem alterar a quantidade de saburra formada, em indivíduos periodontalmente saudáveis.

Palavras-chave: halitose; CSV; flavorizantes; LSS, dentifrícios.

ABSTRACT

Morning bad breath has been correlated with the release of volatile sulphur compounds (VSC) produced in higher concentrations during sleep even in healthy subjects. Thus, the aim of this study was to: (chapter I) evaluate the effects of sodium lauryl sulphate (SLS) present in a commercial dentifrice on morning bad breath and tongue coating formation; and (chapter II) evaluate the effects of a flavour-containing dentifrice on the VSC formation on morning bad breath and tongue coating formation. Both two-step single-blinded, crossover and randomised studies included a total sample of 75 dental students with healthy periodontium. On chapter I, 25 patients were assigned to two experimental groups: dentifrices with (test) or without (control) SLS and on chapter II, 50 patients were assigned to dentifrices with (test) or without (control) flavour. All volunteers received the designated dentifrice and a new toothbrush for a 3-day brushing regimen for two 30-day periods. A seven-day washout interval was included, in which a placebo dentifrice was used. The assessed parameters were: plaque index (PI), gingival index (GI), organoleptic breath (ORG), VSC levels by sulphide monitor before (H1) and after (H2) tongue cleaning, tongue coating wet weigh (TC), the maximum inhibitory dilution of dentifrices and BANA test for tongue coating (chapter II). Inter-group analysis showed that SLS reduced organoleptic scores and H1. Regarding the presence of flavour, inter-group analysis showed a decrease in organoleptic scores and VSC levels (H1/H2) for test group ($p<0.05$). BANA test for tongue coating did not show difference between groups. Within the limits of this study, it could be concluded that SLS-containing dentifrice is able to control VSC formation on morning bad breath in the presence of tongue coating. Nevertheless, when SLS is associated with flavour components in a dentifrice, morning bad breath reduction is observed along with the decrease of VSC levels and organoleptic scores in the whole mouth, regardless of the amount of tongue coating, in periodontally healthy subjects.

Key Words: bad breath; halitosis, VSC; flavour; SLS; dentifrices.

INTRODUÇÃO

A halitose (mau odor bucal, mau hálito, *fetor ex ore*) é uma queixa comum entre adultos de todas as faixas etárias e apresenta natureza multifatorial. Alguns tipos de halitose podem estar relacionados a certos hábitos, como fumar, consumir bebidas alcoólicas, ou à ingestão recente de certos tipos de alimentos. Um segundo tipo de mau hálito envolve a atividade proteolítica da microbiota residente no dorso da língua e nas superfícies dentais (De Boever & Loesche, 1995). O mau odor bucal é causado pela presença de compostos sulfurados voláteis (CSV) no ar exalado, especialmente metil mercaptana (CH_3SH) e gás sulfídrico (H_2S) (Tonzetich 1971,1977), além de ácidos graxos de cadeia curta, como ácido propiônico e ácido valérico, e poliaminas como putrescina e cadaverina (Goldberg *et al*., 1994). Com exceção dos CSV (gás sulfídrico, metilmercaptana e dimetilsulfeto), as demais substâncias podem ser consideradas como contribuidoras menores para o mau odor oral, pois, apesar de presentes, possuem peso molecular elevado, sendo, portanto, menos voláteis no ambiente oral, diminuindo a sua detecção pelo olfato (Tonzetich, 1967). Estima-se que os fatores orais decorrentes da presença de microorganismos na saburra lingual e biofilme dental contribuam em cerca de 90% para os casos de halitose (Tonzetich, 1977).

Ainda não se sabe exatamente quais das 500 a 600 espécies de microorganismos orais, atualmente descritos na literatura, degradam substratos proteicos como peptídeos e aminoácidos, gerando os compostos mal cheirosos (Loesche & Kazor, 2002). Estudos indicam que a microbiota associada à halitose é predominantemente anaeróbia, proteolítica, Gram negativa, a qual utiliza diferentes fontes de nutrientes oriundas do próprio hospedeiro, principalmente células descamadas, saliva e fluido gengival, assim como alimentos da dieta que não foram rapidamente metabolizados ou deglutidos (Persson *et al*., 1990; De Boever & Loesche, 1995).

Alguns estudos demonstraram uma associação entre o desenvolvimento da doença periodontal e o perfil microbiano responsável pela formação dos CSV (Rizzo, 1970; Solis-Gaffar *et al*., 1980; Persson *et al*., 1990). A doença periodontal freqüentemente promove mau odor oral, e a intensidade do odor aumenta com a severidade da doença (Yaegaki & Sanada, 1992). Por outro lado, Rizzo (1970) demonstrou o papel dos sulfidretos como agentes colaboradores para que ocorra a penetração de antígenos bacterianos, como os lipopolissacarídeos, no epitélio gengival saudável, resultando em inflamação. Solis-Gaffar

et al. (1980) demonstraram a correlação positiva entre o volume do fluido gengival e a produção de sulfidretos, aumentando a severidade da inflamação gengival. Além disso, Yaegaki e Sanada (1992) encontraram maiores concentrações de CSV em pacientes que apresentavam bolsas periodontais com profundidades de sondagem maiores que 4 mm. Dessa forma, considera-se que os CSV não somente contribuam na etiologia da halitose, como também possam ser responsáveis por uma série de eventos e efeitos sobre a estrutura e o metabolismo dos tecidos periodontais, propiciando, de certa maneira, o desenvolvimento da doença. Porém, mesmo em indivíduos periodontalmente saudáveis, durante o sono a proliferação de microorganismos bucais associados à hipo-salivação noturna é responsável pela maior produção de CSV, os quais promovem o mau hálito matinal, (Tonzetich, 1971; Miyazaki *et al*., 1995; van Steenbergue *et al*., 2001; Carvalho *et al*., 2004).

Embora a halitose seja uma condição humana comum, afetando principalmente a população adulta (Tonzetich, 1977), poucos estudos epidemiológicos e investigações científicas têm sido feitas sobre esse problema. Uma das razões para isso pode ser a dificuldade de quantificar e monitorar essa condição de uma forma padronizada. Estudos pioneiros que investigaram o mau odor bucal tiveram suas mensurações baseadas em cultura de biofilme dental e exsudatos da bolsa periodontal em meios de cultura bacteriana (Tachibana, 1957), escalas organolépticas subjetivas (Nara, 1977; Tsunoda *et al*., 1981) e medidas quantitativas por meio de cromatografia gasosa (Solis-Gafar, 1975; Tonzetich, 1977; Kostelc *et al*., 1984). A cromatografia gasosa, apesar de ser um método com especificidade para distinguir os gases emanados da cavidade oral (Tonzetich, 1977), apresenta limitações para medir halitose em várias amostras em um curto período de tempo, enquanto que os outros métodos descritos acima apresentam dificuldades de padronização.

Devido a essas limitações, um monitor portátil para os CSV (Halimeter® RH-17E, Interscan Corp., Chatsworth, California, USA) foi desenvolvido e comparado ao exame organoléptico, considerado o padrão ouro das medidas de hálito (Rosenberg *et al*., 1991 a,b). Nesses estudos, os autores compararam a utilização do monitor de sulfetos com exames organolépticos feitos por juízes treinados e encontraram correlação entre os dois métodos ($r = 0{,}603$, $p < 0{,}001$). A partir de então, estudos têm sido realizados com a utilização do monitor portátil de sulfetos (Goldberg *et al*., 1994; Kozlovsky *et al*., 1994;

De Boever & Loesche, 1995; Silwood *et al*., 2001; Carvalho *et al*., 2004). Esse monitor succiona o ar exalado da boca e, quando os CSV atingem o sensor interno do aparelho, ocorre uma reação eletroquímica, e a oxidação dos compostos pode ser proporcionalmente lida como ppb do gás ionizado. A leitura é realizada em partes por bilhão; isto é, 1 grama de CSV para 1 000 000 000 de gramas de ar (Tárzia, 2000). Segundo o fabricante do aparelho, a leitura pode ser interpretada da seguinte maneira: (1) até 80 ppb são considerados valores normais, sem cheiro perceptível; (2) de 80 a 100, o cheiro é perceptível, podendo ser considerado como uma halitose leve; (3) de 100 a 120, halitose moderada; (4) de 120 a 150, halitose mais pronunciada, e (5), acima de 150, halitose severa.

Estratégias para controlar o mau hálito estão relacionadas com o controle do crescimento de bactérias, principalmente as proteolíticas, e envolve o debridamento de dentes e língua em combinação com o uso de agentes antimicrobianos (Loesche & Kazor, 2002). Logo, uma variedade de produtos tem sido utilizada na tentativa de inibir ou mascarar o mau odor bucal, incluindo agentes em gomas de mascar, enxaguatórios e dentifrícios.

Os dentifrícios, em décadas passadas, tinham a função principal de eliminar manchas dos dentes e fornecer à cavidade bucal uma sensação de frescor e limpeza. Com o reconhecimento do sucesso do uso de dentifrícios como veículo do flúor, outros agentes quimioprofiláticos foram acrescentados a eles (Scheie, 1994). Os dentifrícios contêm os seguintes ingredientes essenciais: sistema abrasivo para auxiliar a remoção de manchas, umectante para carregar tanto o abrasivo como o agente quimioprofilático, surfactante para promover espuma e ação detergente, ligador para conceder as propriedades reológicas, e flavorizantes para dar sabor ao dentifrício (Scheie, 1994). Além dos ingredientes essenciais, agentes quimioprofiláticos como pirofosfatos, sais de zinco, cloreto de estrôncio, bicarbonato de sódio e triclosan associado à copolímeros também têm sido acrescentados aos dentifrícios. Dessa forma, os dentifrícios passaram de simples veículos de flúor para agentes quimioterapêuticos, apresentando ação antimicrobiana, anticálculo, anti-hipersensibilidade dentinária dentre outras (Jenkins *et al*., 1991, Brunette *et al*., 1998; Nogueira-Filho *et al*., 2002). Sendo também utilizados para promover hálito agradável e sensação de frescor bucal.

Estudos têm demonstrado a efetividade de dentifrícios contendo diferentes agentes antimicrobianos em controlar o mau hálito: Sharma *et al*. (1999), Niles *et al*. (1999) e Nogueira-Filho *et al*. (2002) demonstraram que dentifrícios contendo triclosan foram capazes de reduzir o mau odor bucal. Já os estudos de Niles *et al*. (1993) e Brunette *et al*. (1998) mostraram a efetividade do bicarbonato de sódio presente em dentifrício na redução dos níveis de CSV, enquanto Gerlach *et al*. (1998) demonstraram que um dentifrício contendo fluoreto estanhoso também foi efetivo para reduzir o mau odor bucal. Alguns autores (Davis, 1978, 1981; Bowen, 1992) sugeriram que a presença de flavorizantes em dentifrícios pode estimular o fluxo salivar, alterando a capacidade de limpeza ou clearance da cavidade bucal, acelerando a eliminação das bactérias por deglutição e alterando, dessa forma, a formação do mau odor bucal. Além disso, Jenkins *et al*. (1991) demonstraram que o lauril sulfato de sódio (LSS) pode apresentar um efeito antimicrobiano e inibidor do biofilme dental e que a presença deste ingrediente pode fornecer substantividade ao dentifrício (Moran *et al*., 1988, Jenkins *et al*., 1990). Sendo assim, a presença do surfactante lauril sulfato de sódio nos dentifrícios poderia alterar a proliferação de bactérias produtoras de CSV.

Estudos recentes de Nogueira-Filho *et al*. (2000, 2002) avaliaram, por meio do modelo da gengivite experimental em humanos, o efeito de dentifrícios contendo triclosan na redução do biofilme dental supragengival, da inflamação gengival e dos CSV. Nesses estudos, os autores observaram uma discreta efetividade na redução dos parâmetros avaliados, mesmo nos dentifrícios considerados controles, sugerindo uma possível ação efetiva de outros componentes dos dentifrícios como o lauril sulfato de sódio e os flavorizantes, sem, todavia, ficar estabelecida qual a participação dessas substâncias na redução dos CSV. No entanto os trabalhos de Nogueira-Filho *et al*. (2000, 2002) avaliaram uma população com gengivite induzida experimentalmente em um quadrante, por 21 dias, sem avaliar os efeitos desses dentifrícios em indivíduos periodontalmente saudáveis, os quais também apresentam mau hálito matinal (van Steenberghe *et al*., 2001; Carvalho *et al*., 2004).

Dessa forma, este trabalho se propôs a avaliar:

1- O efeito do lauril sulfato de sódio (LSS), presente em um dentifrício, no hálito matinal e na saburra lingual de indivíduos periodontalmente saudáveis;

2- O efeito dos flavorizantes, presentes em um dentifrício, no hálito matinal e na saburra lingual de indivíduos periodontalmente saudáveis.

CAPÍTULO I

Effects of sodium lauryl sulphate (SLS) present in dentifrice on morning bad breath and tongue coating in a panel of periodontally healthy subjects

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Abstract

Aim: This study aimed to evaluate the effects of sodium lauryl sulphate (SLS) present in a commercial dentifrice on morning bad breath and tongue coating formation.

Methods: A two-step, single-blinded, crossover, randomised study was carried out in 25 dental students presenting a healthy periodontium. The patients were assigned into two experimental groups: SLS dentifrice (test) and WSLS dentifrice, without sodium lauryl sulphate (placebo). The volunteers received the designated dentifrice and a new toothbrush for a 3x/day brushing regimen for two 30-day periods with no tongue cleaning. A seven-day washout interval was used between the two periods. The assessed parameters were: plaque index (PI), gingival index (GI), organoleptic breath (ORG), VSC levels by sulphide monitor before (H1) and after (H2) tongue cleaning, tongue coating wet weigh (TC) and antimicrobial activity of each dentifrice, determined by their maximum inhibitory dilution (MID).

Results: Intra-group analysis showed a statistical difference in ORG, PI and GI scores for SLS group ($p<0.05$). Inter-group analysis showed lower PI, GI, ORG scores and H1 level ($p<0.05$) only for SLS group; however, no differences were observed for H2 and TC ($p>0.05$). The antimicrobial activity (MID) was detected only in the test group for the assessed microorganisms.

Conclusions: Sodium lauryl sulphate (SLS) present in dentifrice seems to prevent VSC formation in morning bad breath regardless of the amount of tongue coating in healthy subjects.

Keywords: halitosis; VSC; tongue coating; sodium lauryl sulphate; dentifrices.

Introduction

Putrefactive microbial activity within the oral cavity results in the production of volatile sulphur compounds (VSC) including methyl mercaptan, dimethyl sulphide and hydrogen sulphide, as well as other bad smelling compounds, such as putrescine, cadaverine, indole and skatole (Kleinberg & Westbay 1992). These gases originate from the breakdown of amino acids such as cysteine, cystine, methionine, or peptides by microbial putrefaction (Persson et al. 1990).

Jenkins et al. (1991) reported that the substantivity of sodium lauryl sulphate (SLS) might influence the dentifrice antimicrobial activity in the mouth. This substance is used in toothpastes and mouthrinses as an emulsifying and surface cleaning agent. Therapeutic aspects of SLS have been linked to its surface-active properties. Other studies have suggested that SLS binds to bacterial proteins, causing an interference of bacterial attachment to tooth surfaces thus reducing bacterial biofilm formation (Pader 1988, Barkvoll 1991). However, no study has evaluated the possible effects of SLS present in oral care products against malodor. Therefore, the purpose of the present investigation was to evaluate the effect of SLS, present in dentifrice, in the formation of VSC and tongue coating in a panel of periodontally healthy subjects.

Material and Methods

Subjects

Twenty-five volunteers were selected among the under-graduate students attending the School of Dentistry at Piracicaba (UNICAMP, São Paulo, Brazil). Thirteen men and 12 women, between the ages of 25 and 35, who agreed and signed the informed consent to participate on the study, and who presented no systemic disorders were included in the study. The following subjects were excluded: smokers, pregnant women, individuals who had taken antibiotics over the last 6 months or have permanently used any drugs and/or presented otorhinolaryngological and/or respiratory problems. Regarding clinical criteria, individuals wearing appliances or orthodontic contention devices, and presenting deep tongue fissures, dental implants, prostheses or badly adapted restorations were also excluded from the study. In addition, all participants should present normal salivary flow rate (1.5 – 2.5 mL) and at least 24 teeth, which did not present gingival probing depth

greater than 3 mm and gingival index and plaque index (Ainamo & Bay 1975) equal to 1 in more than 10% of sites.

Ethical Aspects

The present study had previously obtained approval by the FOP-UNICAMP Ethical Committee for Human Research (Protocol # 062/2003). The selected volunteers who agreed to participate on the study signed an informed consent, according to the Helsinki II Declaration and the Dentistry Ethical Code (CONEP/MS).

Test and control products

Two dentifrices were compared: a dentifrice containing sodium lauryl sulphate (SLS - Close Up® Red - Unilever, São Paulo, Brazil) and a dentifrice without sodium lauryl sulphate (WSLS - placebo). The SLS dentifrices were bought in stores and the placebo dentifrices were produced and supplied by Unilever (São Paulo, Brazil). Both dentifrice samples (SLS and WSLS) were placed in white new plastic coded tubes in such a way that did not allow direct identification of the product. The codes were not disclosed until the study had been completed.

Study Design

A crossover, randomised, single-blinded study was carried out on a sample of 25 healthy individuals, randomly assigned to two experimental groups: SLS and WSLS. The volunteers received the designated dentifrice and a new toothbrush for a 3x/day brushing regimen for 2 periods of 30 days each (Figure 1). A wash out interval of 7 days occurred between the periods in which all the volunteers used the control dentifrice to avoid a possible carry over effect.

Clinical assessment and pre-experimental phase

The following clinical parameters were evaluated: plaque index (PI), gingival index (GI) (Ainamo & Bay 1975), probing depth (PD), gingival recession (GR) and clinical attachment level (CAL). Seven days prior to the first experimental period, the volunteers were scheduled for an appointment at 7 a.m. for breath analysis, in compliance with the following criteria: the night before the appointment, volunteers were required not to ingest

piquant food, with garlic or onions, and alcoholic beverages. In the morning, volunteers should be in absolute fasting, without performing any type of oral hygiene and should not use any cosmetics that could release odors/perfumes (Rosenberg 1996, Neiders & Ramos 1999). The organoleptic breath measurement (ORG) and VSC levels were obtained by the use of a sulphide monitor (Halimeter®, Interscan, USA) – (H1). Removal and collection of tongue coating (TC) was then performed with tongue scrapers and a new VSC measurement was taken (H2). PI and GI were evaluated and data recorded. Professional removal of calculus using Gracey curettes was performed as well as dental biofilm removal with rubber cups and prophylactic paste. All volunteers received the placebo dentifrice and a new toothbrush (Close Up®, Unilever, SP, Brazil). All volunteers received verbal and written instructions to use the dentifrice three times a day for 7 days. They were also asked to suspend the use of any type of tongue cleaning and the use of mouthrinses. The use of dental floss was not restricted, considering the crossover design of the study, in which individual variables could be compensated.

Experimental phase

After pre-experimental phase, the following parameters were assessed: ORG, H1, H2, TC, PI and GI on days 0 and 30 of each experimental period. According to the crossover protocol, the volunteers received one of the assigned dentifrices and a new toothbrush (Close Up®, Unilever, SP, Brazil) that were used during the following the 30-day periods.

Organoleptic analyses

Individuals were asked to keep their mouths completely closed for three minutes, breathing only through the nose. After this time had elapsed, the volunteer was instructed to release the air slowly by mouth, 10 cm distant from the examiner's nose (Kozlovsky et al. 1994), who was calibrated for this purpose and blind to which group each individual belonged to. A score from 0 to 5 was then attributed according to Rosenberg's (1991) scale: (0) no appreciable odor; (1) barely noticeable odor; (2) slight, but clearly noticeable odor; (3) moderate odor; (4) strong odor; and (5) extremely foul odor.

VSC measurements

Quantitative measurements of VSC were performed with the use of a portable industrial sulphide monitor (Halimeter® RH-17E, Interscan Corp., Chatsworth, California, USA). The monitor was adjusted to zero on environmental air before each measurement and the maximum peak of VSC concentrations was expressed in parts per billion (ppb). Immediately before breath measurement, each patient repeated the procedure of keeping his/her mouth shut for 3 minutes, breathing through the nose. After this period, a disposable flexible drinking straw was connected to the factory-supplied tube and inserted into the subject's mouth, 4 cm behind the incisors, while the mouth was kept slightly opened. The volunteer was instructed to breathe through the nose during this process, so that the monitor vacuum pump could suck the air through the tube (Bosy et al. 1994). Each patient's maximum peak of volatiles was determined in ppb of sulphur compounds through direct reading on the monitor analogical scale. This procedure was carried out in each volunteer at all times before and after tongue coating removal.

Tongue Coating Removal and Collection

The tongue coating was removed by the use of sterile tongue scrapers (Saudbucal®, SP, Brazil) on the posterior third of the patient's tongue. Four back to front movements, were performed to remove the coating, which was deposited in a pre-weighed sterile recipient. The recipient containing the tongue coating was weighed again and a wet weight value was recorded.

Antimicrobial activity of dentifrices against target organism strains

The antimicrobial activity was evaluated by the antimicrobial susceptibility testing of anaerobes modified for oral products (Moran et al. 1988, Wade & Addy 1992). Based on that method, the antimicrobial action of toothpastes was derived from the water-soluble components (Moran & Addy 1984) and supernatants from each formulation were prepared. Thus, stock solutions of toothpaste preparations were made by mixing 10g of toothpaste in 10mL of distilled water for 5 min on a rotatory mixer. The resulting slurries were then centrifuged at 4.000 rpm for 15 minutes; the supernatant was serially diluted with distilled water up to a final dilution of 1:1024. Two milliliters of each dilution was then mixed with 18mL of either molten enriched Trypticase Soy agar (ETSA) supplemented with 5% defibinated sheep blood, hemin, menadione, nitrate, and lactate to

support growth of oral species or Mueller Hinton agar (MH) and poured onto plates giving a range of dilution from 1:10 to 1:20480.

A panel of 17 different organisms was used including odorigenic species from normal oral flora, putative periodontal pathogens and opportunistic pathogens and both reference strains and strains from the departmental collection (strain number prefix) were isolated from a variety of oral infections.

The organisms were suspended in phosphate buffered saline to a MacFarland 0.5 standard turbidity. The plates plus a control plate containing no test agent were inoculated by a multipoint inoculator, which delivered 3 uL of each microbial suspension to the plates. This enabled all 17 microorganisms to be tested simultaneously. The plates were incubated for 48-96 hours at 37ºC under aerobic or anaerobic conditions. The maximum inhibitory dilution (MID) was taken as the highest dilution affording no growth, a single colony, or a fine visible haze. The control plate without any dentifrice was checked for viability of the test organisms. In order to control the study and ensure reproducibility, the experiments were performed on three occasions and the results expressed as the medians values.

Statistical Analysis

Wilcoxon test was applied to the data. For all the analysis, a 5% significance level was established and the data was analyzed using the software BioEstat 3.0 (Ayres et al. 2003).

Results

There was a better acceptance for the use of SLS dentifrice than the placebo one, since the volunteers often complained about the unpleasant feeling with the use of the dentifrice without SLS. However, all 25 individuals completed the research.

Intra-group data

Intra-group results are shown in Tables 1 e 2. There was a reduction on PI, GI and ORG scores on day 30 for the SLS group ($p<0.05$). VSC levels (H1/H2) and TC did not change with treatments ($p>0.05$). However, the WSLS group showed a discrete increase in PI and GI from day 0 to day 30 (from 1.16 ± 1.44 to 1.88 ± 2.23, $p<0.01$ and from 1.88 ± 1.82 to 2.5 ± 2.23, $p<0.05$, respectively). Tongue coating removal resulted in a decrease of VSC level from H1 to H2 only for WSLS group.

Inter-group data

Before treatments (day 0), there were no statistically significant differences ($p>0.05$) for all the parameters among the 25 volunteers at the beginning of each experimental period (Table 3). Significantly lower scores were observed for PI (0.98 ± 1.35) and GI (1.18 ± 1.62) for SLS group when compared to WSLS group (1.88 ± 2.23 and 2.50 ± 2.22, respectively). Lower values in ORG and H1 were found only for SLS group ($p<0.05$). However, the H2 and TC were not affected by the treatments ($p>0.05$). Comparisons between treatments after 30 days are presented in Table 4.

Antimicrobial activity of dentifrices (MID)

The MID values for the two dentifrice formulations are shown in Table 5. The majority of organisms were approximately equally susceptible to SLS toothpaste at the tested concentrations. Exceptions were *P. gingivalis* and *P. intermedia* strains which were more susceptible than the others. WSLS, on the other hand, showed no antimicrobial activity against any of the microorganisms.

Discussion

The use of a toothpaste and toothbrush is the most common form of oral hygiene practiced by individuals in the majority of the countries (Emslie, 1980). Dentifrices provide an ideal vehicle for the carriage of agents that may benefit dental health, notably fluoride, which acts in the prevention of dental caries (Murray 1976). Despite the common use, for many years, of anionic detergents like sodium lauryl sulphate (SLS) in toothpastes, there is little information concerning its antimicrobial and antihalitosis properties. In the present study, the use of a dentifrice containing SLS showed a reduction of organoleptic scores in morning bad breath in the presence of a 30-day tongue coating accumulation. In addition, PI and GI scores were statistically lower in the subjects that used the SLS dentifrice, which could represent a bias since there was a better acceptance for the use of SLS dentifrice than the placebo dentifrice.

Sodium lauryl sulphate is a detergent used in many consumer products (Pader, 1988) and it has been shown to present neither beneficial nor detrimental effects on the clinical manifestation of gingivitis (Baelum et al., 1994), on salivary IgA levels, as well as on the local cellular immune responses, as measured by immunohistological techniques in cells

expressing surface antigens (Schmeiser & Gulzow 1992). Furthermore, no adverse effects on the oral mucosa could be linked to SLS in the concentrations used in toothpastes. This component has a variety of functions that provide both sensorial and therapeutic benefits for toothpastes. The sensorial aspects have been linked to detersive and emulsifying properties of SLS, which imparts a foaming sensation with perception of cleaning. These properties are thought to increase consumer appeal and compliance (Fakhry-Smith et al. 1997), which could be observed in the present study according to the volunteers' comments.

The present study also indicates a reduction of organoleptic scores and VSC levels in the presence of tongue coating (H1) with the use of SLS dentifrice, as well as an *in vitro* antimicrobial activity of SLS tested by MID in a panel of microorganisms. Considering the bacterial-linked aetiology of bad breath, it could be considered that the effect of SLS is related to bacterial protein denaturation (Pader 1988, Barkvoll & Rolla 1989), which may reduce specific bacterial activity in sulphur amino acids, the precursors of VSC.

Several reports (Yaegaki & Sanada 1992, Bosy et al. 1994, De Boever & Loesche 1995, Miyazaki et al. 1995) showed that the amount of tongue coating is closely correlated with malodor and whenever it is removed, the VSC levels are reduced (Yaegaki & Sanada 1992). Lee et al. (2003) investigated the relationship of VSC concentrations to tongue coating and periodontal health. These authors performed gas chromatography to analyse each VSC component from the mouth air sampled before and after tongue scraping. It was then suggested that the composition of tongue coating, including Gram-negative bacteria and sulphur-containing substrates, is more critical for the production of oral bad breath than the amount of tongue coating itself. In the same manner, the present study showed a reduction of malodor's parameters regardless of the amount of tongue coating present.

When tongue coating was scraped (H1 to H2), VSC level reduced only in the WSLS group. This could be explained by the fact that the placebo group showed higher VSC levels than the test group at day 30. When tongue coating was removed, there was a reduction of VSC levels for WSLS. However, these values remained higher than the VSC levels obtained before tongue scraping in the SLS group. Also, plaque and gingival indexes were higher in the placebo group. These data may suggest that in a sample of healthy subjects there are other important halitosis-inducing factors besides tongue coating.

Although tongue biofilm have been implicated as a major source of odor production in subjects with halitosis, its composition is still not well characterized (Kazor et al. 2003). Studies of cultivable tongue microbiota have been limited by the difficulties of in vitro growth techniques, the low percentage of total organisms recovery, and the inadequacy of microbial identification (Hartley et al. 1996). Kazor et al. (1999) were able to recover only up to 30% of the viable microbial count using a growth medium supplemented with human blood and saliva. These findings suggest that much of the tongue microbiota has not yet been cultivated and the use of molecular approaches seems to be mandatory to better characterize the tongue microflora. Furthermore, the microbiota responsible by malodor formation was also not yet well characterized (Loesche & Kazor 2002). In the present study, the SLS tested by MID showed antimicrobial activity against some microrganisms, which is in agreement with Jenkins et al. (1991) who concluded that SLS provides antimicrobial properties to toothpaste formulations. Interestingly, there is no information in the literature describing the possible effects of SLS present in oral care products against oral malodor. This is one of a few studies evaluating the action of a SLS dentifrice on bad breath, and this would be worthy further investigations. Also, more studies are necessary to verify the effect of other dentifrice components in reducing VSC formation in the whole mouth, even in the presence of dental biofilm and tongue coating in periodontally healthy individuals. Within the limits of this study, it could be concluded that SLS-containing dentifrices increase the control of VSC formation in the morning bad breath in healthy subjects.

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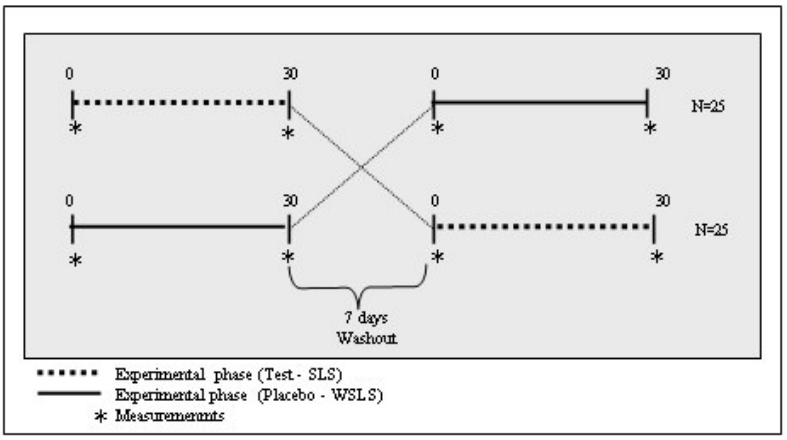

Figure 1: Experimental design

Table 1. Intra-group comparisons between day 0 and 30 for SLS group (mean±SD; n=25).

Measurements	Day 0	Day 30
PI (% mean)	1.17 ± 1.74 ^{A}	0.98 ± 1.35 ^{B}
GI (% mean)	2.24 ± 2.59 ^{A}	1.18 ± 1.62 ^{B}
ORG (score - medians)	3 ^{A}	2 ^{B}
H1(ppb)	117.88 ± 52.29 ^{Aa}	118.76 ± 64.48 ^{Aa}
H2 (ppb)	132.44 ± 65.22 ^{Aa}	116.72 ± 53.59 ^{Aa}
TC (g)	0.18 ± 0.11 ^{A}	0.18 ± 0.10 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 2. Intra-group comparisons between day 0 and 30 for WSLS group (mean±SD; n=25).

Measurements	Day 0	Day 30
PI (% mean)	1.16 ± 1.44 ^{B}	1.88 ± 2.23 ^{A}
GI (% mean)	1.88 ± 1.82 ^{B}	2.50 ± 2.22 ^{A}
ORG (score - medians)	3 ^{A}	3 ^{A}
H1(ppb)	123 ± 66.15 ^{Aa}	192.44 ± 203.29 ^{Aa}
H2 (ppb)	118.28 ± 73.20 ^{Aa}	147.76 ± 108.72 ^{Ab}
TC (g)	0.14 ± 0.08 ^{A}	0.23 ± 0.14 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 3. Inter-group comparisons between day 0 for WSLS/SLS groups (mean±SD; n=25).

Measurements	WSLS	SLS
PI (% mean)	1.16 ± 1.44 ^{A}	1.17 ± 1.74 ^{A}
GI (% mean)	1.88 ± 1.82 ^{A}	2.24 ± 2.59 ^{A}
ORG (score - medians)	3 ^{A}	3 ^{A}
H1(ppb)	123 ± 66.15 ^{Aa}	117.88 ± 52.29 ^{Aa}
H2 (ppb)	118.28 ± 73.20 ^{Aa}	132.44 ± 65.22 ^{Aa}
TC (g)	0.14 ± 0.08 ^{A}	0.18 ± 0.11 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 4. Inter-group comparisons between day 30 for SLS/WSLS groups (mean±SD; n=25).

Measurements	WSLS	SLS
PI (% mean)	1.88 ± 2.23 ^{A}	0.98 ± 1.35 ^{B}
GI (% mean)	2.50 ± 2.22 ^{A}	1.18 ± 1.62 ^{B}
ORG (score - medians)	3 ^{A}	2 ^{B}
H1(ppb)	192.44 ± 203.29 ^{Aa}	118.76 ± 64.48 ^{Ba}
H2 (ppb)	147.76 ± 108.72 ^{Ab}	116.72 ± 53.59 ^{Aa}
TC (g)	0.23 ± 0.14 ^{A}	0.18 ± 0.10 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 5. Antimicrobial activity of dentifrices (WSLS/SLS) against target microorganisms.

Target Organisms	Dentifrices	
	WSLS	SLS
Normal Oral Flora		
S. mutans (ATCC 25175)	no	1:640
S. sobrinus)ATCC 33478)	no	1:80
S. agalactiae (ATCC 13813)	no	1:1280
Putative Periodontal Pathogens		
A. actinomycetemitans (FDC Y4)	no	1:80
A. actinomycetemitans - c.campo 38	no	1:1280
P.gingivalis (ATCC 33527)	no	1:5120
P.intermedia (ATCC 25611)	no	1:2560
F.nucleatum (ATCC 25586)	no	1:1280
Opportunistic Pathogens		
S.typhimurium (ATCC 13311)	no	no
C.freundii (ATCC 8090)	no	no
P.vulgaris (ATCC 13315)	no	no
E. coli (ATCC 25922)	no	no
P. aeruginosa (ATCC 27853)	no	no
S. aureus (ATCC 25923)	no	1:40
E. faecalis (ATCC 10541)	no	1:80
C.albicans (ATCC 1023)	no	1:40
C. krusei (ATCC 6258)	no	1:40

Results shown are medians of three replicates

CAPÍTULO II

Flavour-containing dentifrice can reduce volatile sulphur compounds (VSC) on morning bad breath in a population of healthy subjects.

Journal of Periodontology (submitted)

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Abstract

Background: Morning bad breath has been correlated with the release of volatile sulphur compounds (VSC) produced in higher concentrations during sleep even in healthy subjects. Thus, this study aimed to evaluate the effects of a flavour-containing dentifrice on the formation VSC on morning bad breath.

Methods: A two-step blinded, crossover, randomised study was carried out in 50 dental students with healthy periodontium divided into two experimental groups: flavour-containing dentifrice (test) and non-flavour-containing dentifrice (placebo). The volunteers received the designated dentifrice and a new toothbrush for a 3x/day brushing regimen for 2 periods of 30 days. A seven-day washout interval was used. The assessed parameters were: plaque index (PI), gingival index (GI), organoleptic breath (ORG), VSC levels by sulphide monitor before (H1) and after (H2) cleaning the tongue, tongue coating wet weigh (TC), BANA test from tongue coating samples and the maximum inhibitory dilution (MID) of dentifrices.

Results: The intra-group analysis showed a decrease in organoleptic scores, from 3 to 2, after 30 days for the test group ($p<0.05$). The inter-group analysis showed lower values in PI, GI, ORG, H1 e H2 for test group ($p<0.05$). There was no difference between the amount of TC in test and placebo groups. Regarding BANA test, no correlation was observed among the evaluated parameters. The antimicrobial activity of dentifrices against target organism strains showed the same antimicrobial activity against all the microorganisms.

Conclusions: These findings suggest that a flavour-containing dentifrice is able to reduce morning bad breath in periodontally healthy subjects by decreasing VSC levels, regardless of the amount of tongue coating.

Keywords: bad breath; flavour; VSC; dentifrices.

Introduction

Bad breath or halitosis is caused by the presence of volatile sulphur compounds (VSC) in the exhaled air, especially methyl mercaptan (CH_3SH) and hydrogen sulphide (H_2S) (Tonzetich 1971, Lee et al. 2003) in addition to short-chain organic fatty acid such as propionic acid, butyric acid, valeric acid and polyamines, putrescine, cadaverine and

skatoles (Goldberg et al. 1994). During sleep, the proliferation of oral microorganisms associated with nocturnal hypo-salivation is responsible for the great production of VSC that promotes morning bad breath, even in healthy people (Miyazaki et al. 1995, van Steenbergue et al. 2001, Carvalho et al. 2004). Halitosis is observed in about half of the adult population (Moris & Read 1949) and the literature indicates two approaches to reduce bad breath: cleaning the tongue and the teeth surfaces by physical methods and/or reducing the bacterial loads by chemical agents present in dentifrices and mouthrinses (Loesche & Kazor 2002).

There are many different ingredients in toothpastes, including abrasives, surfactants, flavours, preservatives and humectants. Some authors (Davis 1978, 1981, Bowen 1992) have suggested that the flavour present in dentifrices may influence salivary flow, altering oral cavity clearance, accelerating bacterial elimination by deglutition and thus altering oral malodor formation. However, no studies have yet assessed the possible interference of flavour in oral malodor. Thus, the main objective of this study was to evaluate the effect of flavour present in a dentifrice on the formation of VSC in the morning breath of healthy individuals.

Material and Methods

Subjects

Fifty volunteers were selected among under-graduate and graduate students at the School of Dentistry at Piracicaba (UNICAMP, São Paulo, Brazil). Twenty-seven men and 23 women, between the ages of 18 and 30 (mean 24 years old), who agreed and signed an informed consent, and who presented no systemic disorders were included in this study. The following subjects were excluded: smokers, pregnant women, individuals who had taken antibiotics over the last 6 months or have permanently used any drugs and/or presented otorhinolaryngological and/or respiratory problems. Regarding clinical criteria, individuals wearing appliances or orthodontic contention devices, and presenting deep tongue fissures, dental implants, prostheses or badly adapted restorations were also excluded from the study. In addition, participants in the study should present normal salivary flow rate (1.5 – 2.5 mL) and at least 24 teeth that did not present gingival probing

depth greater than 3 mm and gingival index and plaque index (Ainamo & Bay 1975) equal to 1 in more than 10% of sites.

Ethical Aspects

The present study had previously obtained approval by the FOP-UNICAMP Ethical Committee for Human Research (Protocol # 062/2003). The selected volunteers who agreed to participate on the study signed an informed consent, according to the Helsinki II Declaration and the Dentistry Ethical Code (CONEP/MS, Brazil).

Test and placebo products

Two dentifrices were compared: a flavour-containing test dentifrice (Close Up® Red - Unilever, São Paulo, Brazil) and a non-flavour-containing dentifrice (placebo) that presented the same formulation as the test dentifrice, except for the presence of flavour. The test dentifrices were bought in stores and the placebo dentifrices were produced and supplied by Unilever (São Paulo, Brazil). Both dentifrice samples (test and placebo) were placed in white new plastic coded tubes in such a way that it did not allow direct identification of the product. The codes were not disclosed until the study had been completed.

Study Design

A crossover, randomised, blind study was carried out on a sample of 50 healthy individuals, randomly assigned to two experimental groups: test and placebo dentifrices. The volunteers received the designated dentifrice and a new toothbrush for a 3x/day brushing regimen for 2 periods of 30 days each (figure 1). A wash out interval of seven days occurred between the periods, in which all the volunteers used the placebo dentifrice to avoid a possible carry-over effect.

Clinical assessment and pre-experimental phase

The following clinical parameters were evaluated: plaque index (PI), gingival index (GI) (Ainamo & Bay 1975), probing depth (PD), gingival recession (GR) and clinical attachment level (CAL). Seven days prior to the first experimental period, the volunteers were scheduled for an appointment at 7 a.m. for breath analysis, in compliance with the

following criteria: the night before the appointment, volunteers were required not to ingest piquant food, with garlic or onions, and alcoholic beverages. In the morning, volunteers should be in absolute fasting, without performing any type of oral hygiene and should not use any cosmetics that could release odors/perfumes (Rosenberg 1996, Neiders & Ramos 1999). The organoleptic breath measurement (ORG) and VSC levels were obtained by the use of a sulphide monitor (Halimeter®, Interscan,USA) – (H1). Removal and collection of tongue coating (TC) was then performed with tongue scrapers and a new VSC measurement was taken (H2). PI and GI were evaluated and data recorded. Professional removal of calculus using Gracey curettes was performed as well as dental biofilm removal with rubber cups and prophylactic paste. All volunteers received the placebo dentifrice and a new toothbrush (Close Up®, Unilever, SP, Brazil). All volunteers received verbal and written instructions to use the dentifrice three times a day for 7 days. They were also asked to suspend the use of any type of tongue cleaning and the use of mouthrinses. The use of dental floss was not restricted, considering the crossover design of the study, in which individual variables could be compensated.

Experimental phase

After pre-experimental phase, the following parameters were assessed: ORG, H1, H2, TC, PI and GI on days 0 and 30 of each experimental period. According to the crossover protocol, the volunteers received one of the assigned dentifrices and a new toothbrush (Close Up®, Unilever, SP, Brazil) that were used during the following 30-day periods. Also, TC samples were collected for the BANA test at the beginning and at the end of each experimental period.

Organoleptic analyses

Individuals were asked to keep their mouths completely closed for three minutes, breathing only through the nose. After this time had elapsed, the volunteer was instructed to release the air slowly by mouth, 10 cm distant from the examiner's nose (Kozlovsky et al. 1994), who was calibrated for this purpose and blind to which group each individual belonged to. A score from 0 to 5 was then attributed according to Rosenberg's (1991) scale: (0) no appreciable odor; (1) barely noticeable odor; (2) slight, but clearly noticeable odor; (3) moderate odor; (4) strong odor; and (5) extremely foul odor.

VSC measurements

Quantitative measurements of VSC were performed with the use of a portable industrial sulphide monitor (Halimeter® RH-17E, Interscan Corp., Chatsworth, California, USA). The monitor was adjusted to zero on environmental air before each measurement and the maximum peak of VSC concentrations was expressed in parts per billion (ppb). Immediately before breath measurement, each patient repeated the procedure of keeping his/her mouth shut for 3 minutes, breathing through the nose. After this period, a disposable flexible drinking straw was connected to the factory-supplied tube and inserted into the subject's mouth, 4 cm behind the incisors, while the mouth was kept slightly opened. The volunteer was instructed to breathe through the nose during this process, so that the monitor vacuum pump could suck the air through the tube (Bosy et al. 1994). Each patient's maximum peak of volatiles was determined in ppb of sulphur compounds through direct reading on the monitor analogical scale. This procedure was carried out in each volunteer at all times before and after tongue coating removal.

Tongue Coating Removal and Collection

The tongue coating was removed by the use of sterile tongue scrapers (Saudbucal®, SP, Brazil) on the posterior third of the patient's tongue. Four back to front movements, were performed to remove the coating, which was deposited in a pre-weighed sterile recipient. The recipient containing the tongue coating was weighed again and a wet weight value was recorded.

Enzymatic Assay – BANA Test

Since BANA (N-Benzoyl-DL-Arginine-2-Naphthylamide) test detects trypsin activity produced by several oral proteolytic bacteria, including other odorigenic species such as *Treponema denticola, Porphyromonas gingivalis* and/or *Tanerella forsythensis*, this test provides additional information on the bacterial flora associated with malodor (Kazor et al. 2003). According to Loesche et al. (1990), the BANA reagent card (Knowell, Therapeutic Technologies Inc, Toronto, ON) was used in a portion of the previously weighed tongue coating samples that were placed in an incubator at 55°C for 15 minutes. Results were scored as either blue spots (positive) or no colour change (negative).

Antimicrobial activity of dentifrices against target organism strains

The antimicrobial activity was evaluated by the antimicrobial susceptibility testing of anaerobes modified for oral products (Moran et al. 1988, Wade & Addy 1992). Based on that method, the antimicrobial action of toothpastes was derived from the water-soluble components (Moran & Addy 1984) and supernatants from each formulation were prepared. Thus, stock solutions of toothpaste preparations were made by mixing 10g of toothpaste in 10mL of distilled water for 5 min on a rotatory mixer. The resulting slurries were then centrifuged at 4.000 rpm for 15 minutes; the supernatant was serially diluted with distilled water up to a final dilution of 1:1024. Two milliliters of each dilution was then mixed with 18mL of either molten enriched Trypticase Soy agar (ETSA) supplemented with 5% defibinated sheep blood, hemin, menadione, nitrate, and lactate to support growth of oral species or Mueller Hinton agar (MH) and poured onto plates giving a range of dilution from 1:10 to 1:20480.

A panel of 17 different organisms was used including odorigenic species from normal oral flora, putative periodontal pathogens and opportunistic pathogens and both reference strains and strains from the departmental collection (strain number prefix) were isolated from a variety of oral infections.

The organisms were suspended in phosphate buffered saline to a MacFarland 0.5 standard turbidity. The plates plus a control plate containing no test agent were inoculated by a multipoint inoculator which delivered 3 uL of each microbial suspension to the plates. This enabled all 17 microorganisms to be tested simultaneously. The plates were incubated for 48-96 hours at 37ºC under aerobic or anaerobic conditions. The maximum inhibitory dilution (MID) was taken as the highest dilution affording no growth, a single colony, or a fine visible haze. The control plate without any dentifrice was checked for viability of the test organisms. In order to control the study and ensure reproducibility, the experiments were performed on three occasions and the results expressed as the medians values.

Statistical Analysis

Wilcoxon non-parametric test was applied to the data. Percentages of variation for ORG, H1, H2, and TC for each group were calculated in the following manner: (day 0 minus day 30) x 100 divided by day 0. The results were then analyzed by Wilcoxon test. Correlations

were made among PI, GI, ORG, H1, H2, TC and BANA for all experimental periods using Spearman test. For all the analysis, a 5% significance level was established and data were analyzed using the software BioEstat 3.0 (Ayres et al. 2003).

Results

Intra-group data

Intra-group data are shown in Tables 1 and 2. On day 30, gingival index (GI) significantly reduced in the test group ($p<0.05$). However, the placebo group showed a discrete increase in PI and GI from day 0 to day 30 (from 1.19 $\pm$ 1.75% to 2.40 $\pm$ 2.46%, $p<0.05$). Regarding the organoleptic test (ORG), there was a significant reduction in the scores, from 3 to 2, after 30 days, only for the test group ($p<0.05$). However, VSC levels (H1/H2) and TC suffered no influence from the treatments ($p>0.05$). Tongue coating removal resulted in the increase of VSC level from H1 to H2 only for the placebo group. Additional observations were obtained with Spearman correlation coefficients. These data are presented in Tables 3 and 4.

Inter-group data

Before the treatments (day 0), there were no statistically significant differences ($p>0.05$) for all parameters among the 50 volunteers at the beginning of each experimental period (Table 5). The comparisons between treatments after 30 days demonstrated lower values in PI, GI, ORG, H1 e H2 for the test group ($p<0.05$). There was no difference in the amount of TC between test and placebo groups (Table 6). Using the scores of variations before the treatment as a co-variant, the percentage of changes in ORG scores showed a statistical difference between groups, however no differences were detected for the other evaluated parameters (Table 7).

Regarding BANA test, no correlation was observed among any of the evaluated parameters. Furthermore, when the groups were statistically compared among periods, the presence of flavour did not interfere in the number of BANA +/- results ($p>0.05$).

The antimicrobial activity of the dentifrices, assessed by MID, against target organism strains is shown in Table 8. The test and placebo dentifrices showed the same antimicrobial activity against all the microorganisms.

Discussion

In order to reduce morning bad breath, two approaches have been used and tested in the literature: cleaning the tongue and tooth surfaces by physical methods and/or reducing the bacterial loads by chemical agents present in dentifrices and mouthrinses (van Steenberghe et al. 2001, Nogueira-Filho et al. 2002, Loesche & Kazor 2002, Carvalho et al. 2004). In the present study, the presence of flavours in a commercial dentifrice seemed to reduce morning bad breath in healthy subjects by decreasing the formation of VSC levels. In addition, lower values were observed in the test group for all the evaluated parameters, with the exception of tongue coating, which did not differ between the groups. There are many reports (Yaegaki & Sanada 1992, Bosy et al. 1994, De Boever & Loesche 1995, Miyazaki et al. 1995) showing that the amount of tongue coating is closely correlated with malodor. Miyazaki et al. (1995) evaluated oral malodor using a portable sulphide monitor in 2.672 individuals and observed a high correlation between VSC and tongue coating in all age groups. De Boever & Loesche (1996) examined 16 people who complained of oral malodor and demonstrated that mouth odor was significantly related both to tongue odor and to the amount of coating, which was estimated visually as absent, light, moderate or heavy. In the present study, the amount of tongue coating was determined by wet weigh and was correlated to VSC levels after tongue removal (H2) on the test group and with organoleptic scores in the placebo group. Differently, Miyazaki et al. (1995) and De Boever & Loesche (1996) examined both periodontally compromised and healthy subjects, which may have contributed to the increase in correlation between tongue coating and malodor parameters. It has been demonstrated by Yaegaki & Sanada (1992) that the concentrations of hydrogen sulfide and methyl mercaptan in mouth air were higher in patients with probing depth > 4mm than in healthy individuals. In the present investigation, there was a reduction of malodor levels without a significant decrease in the amount of tongue coating, which is in agreement with Lee et al. (2003). This suggests that the composition of tongue coating, including Gram-negative bacteria and sulphur-containing substrates, seems to be more critical for oral bad breath production than the amount of tongue coating itself.

Also, Yaegaki & Sanada (1992) found that removal of tongue coating markedly reduced VSC production measured by gas chromatography. However, the present results demonstrated that VSC levels, measured after tongue coating removal (H2), increased in

the placebo group, which could be explained by the volatilisation of gases, resulting from the prompt tongue coating removal. However, this increase was not found in the test group, which could indicate that the presence of flavour might be interfering on VSC volatilisation.

According to Rosenberg et al. (1991 a,b), there is a significant correlation between the odor scores measured by judges (ORG) and the VSC levels recorded by a sulphide monitor, which is in agreement with the present data, showing correlation between organoleptic scores and VSC levels measured before tongue coating removal. Goldberg et al. (1994) also reported that, in addition to the sulphurous compounds (H_2S and CH_3SH) measured by the sulphide monitor, there are other evil smelling elements that are not captured by the monitor, and may be perceived by the human olfactory sense. This explains the necessity of using both organoleptic and VSC scores to detect changes in bad breath, as confirmed by Rosenberg & McCulloch (1992), who considered the organoleptic evaluation of breath as the "gold standard" for bad breath measurements.

In the present study, although the placebo group has showed higher values of plaque index and gingival index, this increase did not interfere in neither the amount of tongue coating nor in the BANA test results. BANA test did not show any difference between the treatments, indicating an absence of high loads of proteolytic microorganisms on tongue coating. Also, BANA results did not show a correlation among any of the evaluated parameters, which is in accordance with Kozlovsky et al. (1994), who demonstrated that BANA test was poorly associated with VSC levels. Figueiredo et al. (2002) examined the relationship between the presence of BANA positive species (dental plaque, tongue scraping and saliva) and clinical and oral parameters in healthy and periodontally diseased patients. The authors also did not find any significant correlation between VSC levels and tongue-BANA results. The subjects of the present study were periodontally healthy and did not complain about halitosis, differently from De Boever & Loesche (1995), who demonstrated that tongue coating of individuals with high organoleptic scores was related with high BANA scores in subjects presenting halitosis.

The results of the present investigation indicated a decrease of VSC levels and organoleptic scores with the use of a flavour-containing dentifrice, which is in agreement with others studies (Silwood et al. 2001, van Steenberghe et al. 2001, Carvalho et al. 2004), that postulated that chemical agents had a better effect in reducing bad breath,

primarily due to their efficacy in reducing the load of VSC-related microorganisms and oral debris from niches of the whole mouth, rather than only from supragingival biofilm or tongue coating. In the present study, the test and placebo dentifrices showed the same *in vitro* antimicrobial activity against all the tested microorganisms. This shows that the presence of flavour in the dentifrice may reduce the malodor regardless of the amount of tongue coating and the antimicrobial activity of dentifrices.

Few studies suggest some possible effects of flavours present in oral care products on oral malodor. Moris & Read (1949) reported that dentifrices did have the effect of reducing oral malodor; however this effect disappeared in less than an hour and a half. On the other hand, Brunette et al. (1998) suggested that the presence of flavours could mask the presence of VSC. Other authors (Davis 1978, 1981, Bowen 1992) suggested that flavour may act by altering oral cavity clearance by inducing a fast and passing increase in the salivary flow immediately after the use of a dentifrice. As a matter of fact, this increase could accelerate and eliminate the bacteria by deglutition, thus reducing bad breath. However, further studies are necessary to explain the activity patterns or the influence of flavoured dentifrices and morning breath. Within the limitations of this study, it was concluded that the use of flavour-containing dentifrices seems to reduce morning bad breath in periodontally healthy subjects, by decreasing VSC levels, regardless of the amount of tongue coating.

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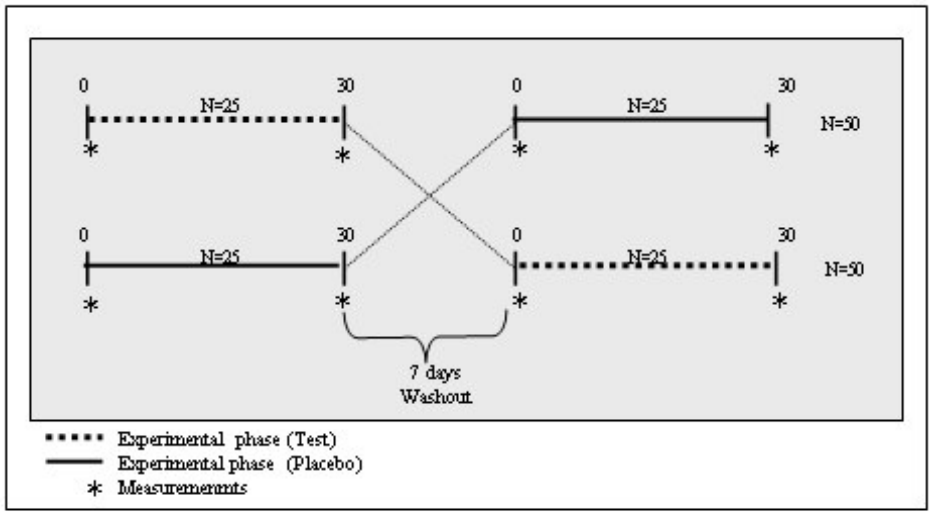

Figure 1: Experimental design

Table 1. Intra-group comparisons between day 0 and day 30 for test group (mean±SD; n=50).

Measurements	Day 0	Day 30
PI (% mean)	1.12 ± 1.61 ^{A}	1.09 ± 1.48 ^{A}
GI (% mean)	2.31 ± 2.57 ^{A}	1.82 ± 2.29 ^{B}
ORG (score - medians)	3 ^{A}	2 ^{B}
H1(ppb)	129.2 ± 73.4 ^{Aa}	132.6 ± 89.7 ^{Aa}
H2 (ppb)	130.9 ± 73.4 ^{Aa}	141.9 ± 131 ^{Aa}
TC (g)	0.2 ± 0.17 ^{A}	0.17 ± 0.11 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 2. Intra-group comparisons between day 0 and day 30 for placebo group (mean±SD; n=50).

Measurements	Day 0	Day 30
PI (% mean)	1.19 ± 1.75 ^{B}	2.40 ± 2.46 ^{A}
GI (% mean)	2.14 ± 2.35 ^{B}	3.05 ± 3.11 ^{A}
ORG (score - medians)	3 ^{A}	3 ^{A}
H1(ppb)	171.7 ± 162.4 ^{Aa}	173.92 ± 98.1 ^{Ab}
H2 (ppb)	169.7 ± 145.2 ^{Aa}	197.5 ± 130.5 ^{Aa}
TC (g)	0.18 ± 0.10 ^{A}	0.21 ± 0.11 ^{A}

Means followed by distinct capital letters in columns differ statistically ($p<0.05$).
Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 3. Spearman correlation for test group at the end of experimental period

	GI	ORG	H1	H2	TC	BANA
PI	r = 0.5487 p= 0	r = 0.1522 p>0.05	r = 0.2584 p>0.05	r = 0.2821 p= 0.0471	r = 0.2406 p>0.05	r = -0.3183 p>0.05
GI		r = 0.2486 p>0.05	r = 0.3232 p= 0.022	r = 0.2965 p= 0.0365	r = 0.0848 p>0.05	r = -0.0058 p>0.05
ORG			r = 0.332 p= 0.0184	r = 0.249 p>0.05	r = 0.263 p>0.05	r = 0 p>0.05
H1				r = 0.7271 p= 0	r = 0.3763 p>0.05	r = -0.3455 p>0.05
H2					r = 0.3365 p= 0.0168	r = -0.2378 p>0.05
TC						r = -0.1132 p>0.05

Table 4. Spearman correlation for placebo group at the end of experimental period

	GI	ORG	H1	H2	TC	BANA
PI	r = 0.3312 p= 0.0187	r = 0.1553 p>0.05	r = 0.1629 p>0.05	r = 0.2041 p>0.05	r = 0.0431 p>0.05	r = -0.4666 p>0.05
GI		r = -0.0579 p>0.05	r = 0.0946 p>0.05	r = 0.114 p>0.05	r = 0.1343 p>0.05	r = -0.2886 p>0.05
ORG			r = 0.4333 p= 0.0017	r = 0.4061 p= 0.0034	r = 0.3927 p= 0.0048	r = -0.3372 p>0.05
H1				r = 0.7741 p= 0	r = 0.1003 p>0.05	r = -0.2221 p>0.05
H2					r = -0.0624 p>0.05	r = -0.2221 p>0.05
TC						r = -0.2998 p>0.05

Table 5. Inter-group comparisons at day 0 between test and placebo groups (mean±SD; n=50).

Measurements	Placebo	Test
PI (% mean)	1.19 ± 1.75 ^{A}	1.12 ± 1.61 ^{A}
GI (% mean)	2.14 ± 2.35 ^{A}	2.31 ± 2.57 ^{A}
ORG (score - medians)	3 ^{A}	3 ^{A}
H1(ppb)	171.7 ± 162.4 ^{Aa}	129.2 ± 73.4 ^{Aa}
H2 (ppb)	169.7 ± 145.2 ^{Aa}	130.9 ± 73.4 ^{Aa}
TC (g)	0.18 ± 0.10 ^{A}	0.2 ± 0.17 ^{A}

Means followed by distinct capital letters in columns differ statistically (p<0.05).
Means followed by distinct lower letters in lines differ statistically (p<0.05).

Table 6. Inter-group comparisons at day 30 between test and placebo groups (mean±SD; n=50).

Measurements	Placebo	Test
PI (% mean)	2.40 ± 2.46 ^{A}	1.09 ± 1.48 ^{B}
GI (% mean)	3.05 ± 3.11 ^{A}	1.82 ± 2.29 ^{B}
ORG (score - medians)	3 ^{A}	2 ^{B}
H1(ppb)	173.92 ± 98.1 ^{Ab}	132.6 ± 89.7 ^{Ba}
H2 (ppb)	197.5 ± 130.5 ^{Aa}	141.9 ± 131 ^{Ba}
TC (g)	0.21 ± 0.11 ^{A}	0.17 ± 0.11 ^{A}

Means followed by distinct capital letters in columns differ statistically (p<0.05).
Means followed by distinct lower letters in lines differ statistically (p<0.05).

Table 7. Percentage of changes (%) among parameters before and after each treatment (mean ± SD; n=50)

	% of changes			
Treatments	ORG	H1	H2	TC
Test	33.33 [a]	-2.69 [a]	-8.40 [a]	15 [a]
Control	0 [b]	-1.28 [a]	-16.19[a]	-16.66 [a]

Means followed by distinct lower letters in lines differ statistically ($p<0.05$).

Table 8. Antimicrobial activity of dentifrices (test/placebo) against target microorganisms.

Target Organisms	Dentifrices	
	Test	**Placebo**
Normal Oral Flora		
S. mutans (ATCC 25175)	1:640	1:640
S. sobrinus)ATCC 33478)	1:80	1:80
S. agalactiae (ATCC 13813)	1:1280	1:1280
Putative Periodontal Pathogens		
A. actinomycetemitans (FDC Y4)	1:80	1:80
A. actinomycetemitans - c.campo 38	1:1280	1:1280
P.gingivalis (ATCC 33527)	1:5120	1:5120
P.intermedia (ATCC 25611)	1:2560	1:2560
F.nucleatum (ATCC 25586)	1:1280	1:1280
Opportunistic Pathogens		
S.typhimurium (ATCC 13311)	-	-
C.freundii (ATCC 8090)	-	-
P.vulgaris (ATCC 13315)	-	-
E. coli (ATCC 25922)	-	-
P. aeruginosa (ATCC 27853)	-	-
S. aureus (ATCC 25923)	1:40	1:40
E. faecalis (ATCC 10541)	1:80	1:80
C.albicans (ATCC 1023)	1:40	1:40
C. krusei (ATCC 6258)	1:40	1:40

Results shown are medians of three replicates

DISCUSSÃO

Estratégias para controlar o mau hálito estão relacionadas com o controle do crescimento de bactérias (Loesche & Kazor, 2002) e, com esse objetivo, uma variedade de produtos tem sido utilizada na tentativa de inibir ou mascarar o mau odor bucal. Os dentifrícios disponíveis no mercado apresentam um grande apelo para a redução ou eliminação do mau hálito e para a promoção de refrescância bucal. Sabendo da abrangência e penetração dos dentifrícios no mercado atualmente, considera-se que eles são uma boa alternativa no controle da halitose. Como foi observado no presente estudo, os resultados gerais dos trabalhos relacionados nos capítulos I e II, quando analisados em conjunto, sugerem que constituintes dos dentifrícios como o LSS e os flavorizantes podem exercer um efeito suficiente para mascarar ou reduzir a presença do mau hálito matinal em indivíduos periodontalmente saudáveis. Porém outros possíveis ingredientes, combinações de ingredientes e variações de formulações tornam a avaliação clínica de dentifrícios extremamente difícil (Jenkins *et al*., 1990) no que tange ao controle de variáveis para evitar possíveis vieses.

No capítulo I, o LSS apresentou *in vitro* atividade antimicrobiana frente a uma amostra de microorganismos testados, o que está de acordo com os trabalhos de Addy *et al*. (1983) e de Jenkins *et al*. (1991), os quais demonstraram propriedades antimicrobianas do LSS. No presente estudo, entretanto, a atividade antimicrobiana do LSS não foi suficiente para reduzir quantitativamente a saburra lingual formada. Embora a presença do LSS e dos flavorizantes tenha promovido uma redução dos escores organolépticos e dos níveis de CSV, medidos pelo monitor de sulfetos, esses ingredientes não promoveram redução quantitativa da saburra lingual entre os grupos. Estudos demonstraram que a principal fonte de mau odor bucal é a saburra lingual (Tonzetich, 1977; De Boever & Loesche, 1995; Lee *et al*., 2003) e que a remoção da mesma reduz os níveis de CSV (Yaegaki & Sanada, 1992). No presente estudo, porém, quando comparamos os níveis de CSV medidos antes e após a remoção da saburra, dentro do mesmo grupo, encontramos resultados conflitantes. No capítulo I, a remoção da saburra promoveu redução dos CSV no grupo WSLS e, no capítulo II, com a remoção da saburra houve um aumento dos níveis de CSV no grupo placebo, medidos imediatamente após essa remoção. Porém, quando analisamos os grupos testes dos dois capítulos, observamos uma estabilidade nos

parâmetros do hálito (H1/H2). Esses resultados demonstram que há um efeito benéfico da associação dos flavorizantes com o LSS, pois o uso do dentifrício, com ambos os ingredientes, promoveu melhor controle do hálito matinal, independente da presença da saburra lingual. Isso sugere que o dentifrício age na boca como um todo, promovendo redução ou mascaramento do hálito, não somente por agir na saburra lingual.

Loesche & Kazor (2002) afirmaram que a maioria dos casos de mau odor bucal é devido à atividade proteolítica bacteriana e que três principais espécies (*Treponema denticola*, *Porphyromonas gingivalis* e *Tannerella forshytensis*) podem estar relacionadas a essa atividade (Persson *et al*., 1990). Esses microorganismos podem ser detectados de amostras de saburra lingual pelo teste BANA (Loesche *et al*., 1990). Estudos que utilizaram indivíduos com halitose demonstraram que as amostras de saburra lingual apresentaram atividade BANA positiva (Bosy *et al*., 1994; De Boever & Loesche, 1995; Figueiredo *et al.*, 2002) e que a saburra lingual de indivíduos com altos escores organolépticos estava relacionada com maior atividade BANA positiva. Entretanto os resultados do nosso estudo (capítulo II) não apresentaram correlação dos resultados do teste BANA com os escores organolépticos e com os níveis de CSV. Esses resultados vão de encontro aos de Kozlovsky *et al*. (1994) que, embora tenham encontrado correlação entre teste BANA e escores organolépticos, não demonstraram a mesma correlação entre o teste e os compostos sulfurados voláteis. Nossos resultados podem estar relacionados com a diversidade da microbiota bucal relacionada à halitose (Persson *et al*., 1990) que vai além dos microorganismos acessados pelo teste BANA e pela MID e também devido à presença de outros compostos mal cheirosos como ácidos graxos de cadeia curta (ácido propiônico e ácido valérico) e poliaminas como putrescina e cadaverina presentes no hálito (Goldberg *et al*. 1994). Também se deve levar em consideração que as amostras de nossos estudos consistiam de indivíduos periodontalmente saudáveis e sem queixa de halitose, diferentemente das amostras dos estudos acima citados (Kozlovsky *et al*., 1994; De Boever & Loesche, 1995; Figueiredo *et al*., 2002).

Seria interessante, porém, a avaliação de outros dentifrícios contendo diferentes flavorizantes, em combinação ou isolados, respeitando sempre a complexidade da formulação dos dentifrícios e seu efeito associado à escovação dental. De uma forma geral, nossos estudos demonstraram que a presença de ingredientes como o LSS e os flavorizantes nas formulações de dentifrícios, especialmente quando combinados, são

eficazes no controle do mau hálito matinal, após 30 dias de acúmulo de saburra, interferindo na formação dos CSV, em indivíduos periodontalmente saudáveis.

CONCLUSÕES

Diante dos objetivos do presente estudo, conclui-se que:

- O lauril sulfato de sódio presente no dentifrício testado foi capaz de reduzir os escores organolépticos e os níveis de CSV do hálito matinal, antes da remoção da saburra (H1), porém não interferiu na formação de saburra lingual, apesar de ter apresentado atividade antimicrobiana *in vitro*;
- Os flavorizantes presentes no dentifrício exerceram um efeito redutor nos escores organolépticos e nos níveis de CSV do hálito matinal, sem, no entanto, alterar a quantidade de saburra lingual e a qualidade da mesma, medida pelo teste BANA.

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ANEXOS

Dados - Capítulo I:

GRUPO SLS - DIA 0							GRUPO SLS - DIA 30						
AMOSTRA	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**	**AMOSTRA**	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**
1	0	0	3	98	159	0.137	1	0.89	0	3	86	83	0.091
2	0	0	2	63	102	0.301	2	0	0	2	110	107	0.246
3	0.89	4.46	2	89	84	0.167	3	2.67	0.89	2	84	92	0.301
4	0	3.12	2	113	128	0.31	4	2.67	0	2	58	63	0.169
5	0	4.46	3	176	168	0.101	5	0	0	2	109	83	0.181
6	0	0	2	70	57	0.042	6	0	0	2	195	134	0.065
7	0	0	2	83	65	0.041	7	0	0	2	80	70	0.075
8	4.46	2.5	3	83	95	0.2	8	0	2.5	2	156	232	0.148
9	0	0	3	132	124	0.215	9	0	0	2	70	90	0.118
10	4.68	5.46	3	78	123	0.285	10	1.56	1.56	2	111	116	0.215
11	1.78	0	2	118	101	0.118	11	0	0	3	115	118	0.139
12	0	0	2	88	119	0.374	12	0.89	0	3	121	185	0.362
13	0	0.89	3	110	124	0.058	13	0	0.89	2	77	77	0.159
14	0	0.89	3	69	90	0.125	14	0.89	0.89	3	126	231	0.197
15	0	0	2	136	189	0.075	15	0	0	2	85	88	0.121
16	0	0	3	106	169	0.279	16	0	0	2	59	68	0.123
17	0	7.03	3	155	115	0.307	17	0.89	3.9	3	86	87	0.433
18	0	0	2	86	87	0.174	18	0	0	2	93	65	0.115
19	0.89	0.78	3	227	195	0.107	19	0	0.78	2	299	108	0.157
20	4.68	7.03	2	181	81	0.005	20	1.56	4.68	2	65	89	0.051
21	1.78	3.57	4	178	248	0.252	21	0.89	1.78	3	212	256	0.169
22	2.5	4.16	2	80	73	0.046	22	3.33	4.16	2	113	129	0.052
23	3.33	6.66	5	96	194	0.245	23	4.03	4.16	4	286	110	0.294
24	4.16	5	2	264	344	0.122	24	4.16	3.33	2	93	113	0.199
25	0	0	3	68	77	0.295	25	0	0	2	80	124	0.377
Média	1.166	2.2404		117.88	132.44	0.17524	Média	0.9772	1.1808		118.76	116.72	0.18228
DP	1.735331	2.586301		52.29111	65.217	0.105384	DP	1.351664	1.617508		64.4808	53.58601	0.102793
Mediana			3				Mediana			2			

GRUPO WSLS - DIA 0							GRUPO WSLS - DIA 30						
AMOSTRA	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**	**AMOSTRA**	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**
1	0.89	0	4	126	155	0.082	1	1.56	0.78	3	109	200	0.21
2	0	0	3	76	73	0.248	2	0	0	3	80	112	0.42
3	1.78	2.67	3	59	51	0.148	3	3.57	4.46	2	80	76	0.142
4	0	3.22	2	55	48	0.084	4	0	4.46	2	91	60	0.17
5	0.89	2.67	4	301	348	0.154	5	1.78	3.22	3	216	164	0.285
6	0	0	2	93	70	0.113	6	0	0	2	152	142	0.162
7	0	0	3	75	94	0.174	7	0	0	3	109	62	0.104
8	1.66	3.33	3	165	93	0.165	8	2.5	4.16	4	186	174	0.351
9	0	0	2	118	114	0.003	9	0	0	3	101	102	0.254
10	3.12	4.68	4	160	115	0.335	10	4.68	6.25	2	96	50	0.224
11	0	1.78	3	151	140	0.125	11	2.67	1.78	3	92	90	0.196
12	0	0	2	91	214	0.07	12	0.89	0	5	1083	488	0.245
13	0.89	0	3	111	53	0.11	13	1.78	0.89	3	104	98	0.063
14	1.78	3.57	3	185	219	0.185	14	8.92	3.57	3	275	430	0.611
15	0	0	4	248	67	0.177	15	0	0	3	99	90	0.034
16	0	0.89	3	160	84	0.198	16	0	2.34	2	131	129	0.29
17	0	3.9	3	51	114	0.202	17	0	5.46	3	175	105	0.299
18	0.89	2.34	2	47	45	0.056	18	0	5.46	3	91	81	0.097
19	0	0	2	87	132	0.113	19	0	0.78	2	290	296	0.36
20	2.34	1.56	3	57	65	0.129	20	3.12	1.56	2	86	78	0.088
21	3.9	3.12	4	179	228	0.2	21	3.12	3.12	3	375	203	0.195
22	2.5	3.33	2	84	72	0.048	22	4.16	3.33	2	112	113	0.061
23	4.16	5	3	107	109	0.065	23	4.03	6.66	5	195	121	0.375
24	4.16	5	2	71	61	0.025	24	4.16	4.16	3	331	86	0.134
25	0	0	3	218	193	0.301	25	0	0	4	152	144	0.38
Média	1.1584	1.8824		123	118.28	0.1404	Média	1.8776	2.4976		192.44	147.76	0.23
DP	1.4433	1.822083		66.1526	73.20492	0.081418	DP	2.229924	2.22213		203.2859	108.7245	0.136185
Mediana			3				Mediana			3			

Dados - Capítulo II:

GRUPO TESTE - DIA 0							GRUPO TESTE - DIA 30						
AMOSTRA	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**	**AMOSTRA**	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**
1	0	0	3	63	55	0.103	1	0	0	2	57	52	0.03
2	0	0	3	84	94	0.129	2	0	0	2	80	123	0.146
3	0	1.66	4	180	95	0.216	3	0	0	3	63	59	0.054
4	1.56	0.78	3	104	93	0.294	4	0	0.78	3	110	83	0.223
5	0	2.34	3	83	122	0.14	5	3.12	2.34	2	184	261	0.162
6	0	0	3	54	186	0.444	6	0	0	3	53	55	0.066
7	5.35	6.25	4	114	156	0.128	7	3.57	9.82	4	157	120	0.168
8	3.57	4.46	3	178	133	0.125	8	2.67	4.46	3	459	901	0.137
9	0	0	3	160	187	0.072	9	0	0	2	140	201	0.147
10	0	6.25	4	256	65	0.289	10	0	5.35	3	148	124	0.289
11	0	3.12	3	57	54	0.12	11	1.56	2.34	2	73	71	0.077
12	0	5.35	3	156	103	0.165	12	0	4.46	2	97	63	0.032
13	0	3.9	1	64	40	0.02	13	0	3.9	3	110	106	0.126
14	1.78	0	4	284	111	0.2	14	0	0.89	3	66	176	0.133
15	3.22	2.34	3	68	76	0.046	15	1.6	2.34	3	431	143	0.099
16	0	1.61	4	459	429	1.049	16	0.8	2.41	3	334	466	0.548
17	2.67	1.78	4	172	50	0.388	17	3.57	2.67	2	91	120	0.164
18	0	3.57	3	83	80	0.039	18	0	4.46	3	93	130	0.054
19	2.67	4.46	3	123	93	0.054	19	3.57	5.35	3	75	89	0.128
20	0	0	3	94	204	0.159	20	0	0	2	90	114	0.007
21	0.89	1.78	4	195	239	0.494	21	0	0.89	3	139	142	0.322
22	2.67	9.8	3	89	111	0.381	22	5.35	8.03	2	135	125	0.179
23	0	0	2	140	137	0.009	23	0	0	3	97	77	0.239
24	1.78	0	3	126	120	0.226	24	2.67	0.89	3	146	152	0.175
25	0.89	0	4	125	203	0.089	25	1.78	0	3	235	226	0.207
26	0	0	3	98	159	0.137	26	0.89	0	3	86	83	0.091
27	0	0	2	63	102	0.301	27	0	0	2	110	107	0.246
28	0.89	4.46	2	89	84	0.167	28	2.67	0.89	2	84	92	0.301
29	0	3.12	2	113	128	0.31	29	2.67	0	2	58	63	0.169
30	0	4.46	3	176	168	0.101	30	0	0	2	109	83	0.181
31	0	0	2	70	57	0.042	31	0	0	2	195	134	0.065
32	0	0	2	83	65	0.041	32	0	0	2	80	70	0.075
33	4.46	2.5	3	83	95	0.2	33	0	2.5	2	156	232	0.148
34	0	0	3	132	124	0.215	34	0	0	2	70	90	0.118
35	4.68	5.46	3	78	123	0.285	35	1.56	1.56	2	111	116	0.215
36	1.78	0	2	118	101	0.118	36	0	0	3	115	118	0.139
37	0	0	2	88	119	0.374	37	0.89	0	3	121	185	0.362
38	0	0.89	3	110	124	0.058	38	0	0.89	2	77	77	0.159
39	0	0.89	3	69	90	0.125	39	0.89	0.89	3	126	231	0.197
40	0	0	2	136	189	0.075	40	0	0	2	85	88	0.121
41	0	0	3	106	169	0.279	41	0	0	2	59	68	0.123
42	0	7.03	3	155	115	0.307	42	0.89	3.9	3	86	87	0.433
43	0	0	2	86	87	0.174	43	0	0	2	93	65	0.115
44	0.89	0.78	3	227	195	0.107	44	0	0.78	2	299	108	0.157
45	4.68	7.03	2	181	81	0.005	45	1.56	4.68	2	65	89	0.051
46	1.78	3.57	4	178	248	0.252	46	0.89	1.78	3	212	256	0.169
47	2.5	4.16	2	80	73	0.046	47	3.33	4.16	2	113	129	0.052
48	3.33	6.66	5	96	194	0.245	48	4.03	4.16	4	286	110	0.294
49	4.16	5	2	264	344	0.122	49	4.16	3.33	2	93	113	0.199
50	0	0	3	68	77	0.295	50	0	0	2	80	124	0.377
Média	1.12	2.31		129.16	130.94	0.20	Média	1.09	1.82		132.64	141.94	0.17
DP	1.61	2.57		73.39	73.37	0.17	DP	1.48	2.29		89.74	130.96	0.11
Mediana			3				Mediana			2			

GRUPO PLACEBO - DIA 0							GRUPO PLACEBO - DIA 30						
AMOSTRA	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**	**AMOSTRA**	**IP %**	**IG %**	**ORG**	**H1**	**H2**	**TC**
1	0	0	2	111	107	0.003	1	0	0	2	80	83	0.08
2	0	0	2	259	229	0.232	2	0	0	2	196	141	0.253
3	0.83	1.66	3	90	98	0.064	3	2.34	5.46	3	176	178	0.231
4	0	0.78	3	120	138	0.182	4	1.56	0.78	2	85	87	0.128
5	2.34	4.68	1	87	84	0.068	5	5.46	6.25	2	151	153	0.157
6	0	0	4	178	129	0.273	6	3.57	0	3	111	95	0.465
7	5.35	6.25	4	247	153	0.183	7	5.35	10.71	4	117	157	0.401
8	4.46	2.67	3	201	183	0.154	8	2.67	4.46	3	271	175	0.221
9	0	0	3	60	75	0.13	9	0	0	2	91	99	0.053
10	0	6.25	3	324	281	0.197	10	0	3.57	3	298	381	0.238
11	0.78	3.9	2	64	72	0.097	11	0.78	3.12	2	62	68	0.318
12	0	5.35	2	127	193	0.059	12	0	6.25	2	144	161	0.138
13	0.89	3.57	2	54	55	0.19	13	0	3.9	3	100	109	0.282
14	0	0	3	121	124	0.12	14	2.41	0	4	192	266	0.17
15	3.9	1.6	3	73	70	0.147	15	3.22	1.6	3	100	334	0.086
16	0	1.6	4	147	317	0.232	16	2.41	1.61	4	371	263	0.398
17	1.78	1.78	3	64	171	0.042	17	2.67	1.78	2	97	210	0.161
18	0	5.35	3	47	95	0.105	18	0	6.25	2	134	144	0.131
19	4.46	7.14	3	96	95	0.138	19	5.35	6.25	2	130	113	0.166
20	0	0	2	160	146	0.106	20	0	0	2	320	372	0.216
21	2.67	0.89	4	234	314	0.407	21	4.46	0.89	4	326	208	0.306
22	6.25	7.14	2	112	112	0.164	22	9.8	10.71	2	148	142	0.247
23	0	0	3	109	86	0.551	23	0	0	3	139	143	0.326
24	2.67	5.35	3	146	72	0.206	24	3.57	10.71	3	156	165	0.136
25	0	0	4	156	232	0.151	25	4.46	0	4	138	176	0.268
26	0	0	3	203	182	0.076	26	1.56	0.78	3	138	158	0.113
27	0	0	3	115	101	0.295	27	0	0	3	57	59	0.203
28	1.78	2.67	2	132	124	0.354	28	4.46	3.57	2	99	105	0.232
29	0	0	2	64	76	0.102	29	0	7.8	2	72	107	0.177
30	0	0.89	3	134	137	0.193	30	2.67	0.89	3	126	113	0.266
31	0	0	3	220	427	0.174	31	0	0	3	285	297	0.118
32	0	0	3	550	518	0.329	32	2.67	0	3	220	384	0.134
33	1.78	2.5	3	203	322	0.202	33	2.5	7.5	3	130	169	0.309
34	0	0	3	156	159	0.194	34	0	0	3	126	141	0.23
35	3.12	1.56	2	151	138	0.224	35	4.68	6.25	3	190	140	0.419
36	0	1.78	2	78	103	0.119	36	3.57	1.78	3	118	116	0.14
37	0	0.89	2	72	57	0.353	37	0.89	1.56	3	114	150	0.505
38	0.89	0	2	73	99	0.101	38	9.82	0	2	162	187	0.121
39	0	1.78	3	190	145	0.121	39	0.89	1.78	3	169	138	0.194
40	0	0	3	149	162	0.204	40	0.89	0	3	153	129	0.194
41	0	0	3	100	117	0.239	41	0	3.12	3	113	110	0.197
42	0	3.9	3	240	125	0.257	42	0.89	4.68	3	214	415	0.191
43	0	0	2	54	62	0.082	43	0	3.9	2	188	198	0.158
44	0	0.89	3	142	198	0.137	44	0.78	2.34	3	245	290	0.138
45	2.34	4.68	2	113	112	0.145	45	5.64	2.34	2	115	173	0.073
46	1.78	4.68	4	992	942	0.305	46	5.46	4.68	3	362	288	0.101
47	2.5	3.33	2	124	125	0.045	47	3.33	3.33	2	101	108	0.06
48	5	6.66	4	157	113	0.187	48	4.03	6.66	5	524	701	0.311
49	4.16	5	4	658	132	0.266	49	5	5	4	410	638	0.155
50	0	0	2	129	192	0.279	50	0	0	3	132	138	0.11
Média	1.19	2.14		171.72	169.98	0.18	Média	2.40	3.05		173.92	197.50	0.21
DP	1.75	2.35		162.42	145.21	0.10	DP	2.46	3.11		98.13	130.46	0.11
Mediana			3				Mediana			3			