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Cariogenic potential of cows', human and infant formula milks and effect of fluoride supplementation

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The aim of the present study was to evaluate the cariogenicity of cows', human and infant formula milks, supplemented or not with fluoride, in rats. Sixty female Wistar rats were desalivated and infected with *Streptococcus sobrinus* 6715.Animals were divided into six groups: group 1, sterilised deionised distilled water (SDW; negative control); group 2, 5 % sucrose added to SDW (positive control); group 3, human milk; group 4, cows' milk; group 5, Ninho® formula reconstituted with SDW; group 6, Ninho® formula reconstituted with 10 parts per million F and SDW. At day 21 the animals were killed and their jaws removed to quantify total cultivable microbiota, *Strep. sobrinus* and dental caries. The concentration of carbohydrate and fluoride in the milks was analysed. The Kruskal–Wallis test ($\alpha = 5\%$) was used to analyse the data. The caries score by the milk formula was as high as that provoked by sucrose. Regarding smooth-surface caries, human milk was statistically more cariogenic than cows' milk, which did not differ from the SDW and the Ninho® with fluoride ($P>0{\cdot}05$). Groups 2–6 showed higher *Strep. sobrinus* counts when compared with the negative control group ($P<0{\cdot}05$) but no statistically significant difference was found among them ($P>0{\cdot}05$). HPLC analysis showed that infant formula had 9·3 % sucrose and 3·6 % reducing sugars. The infant formula should be considered cariogenic due to the sugars found in it, but fluoride supplementation reduced its cariogenic effect. The human milk was more cariogenic than the cows' milk but not as much as the formula milk.

Caries: Commercial milk formula: Rats

Although it can be preventable, dental caries is considered a multi-factorial infectious and transmissible disease as well as one of the most common chronic diseases of early childhood[1,2]. Sugar-containing solutions fed to infants before sleep time might lead to tooth decay[3,4]. This practice seems to be one of the major causes of heavy infections on tooth surfaces by *Streptococcus mutans*, composing 60 % of the microbial population present in dental plaque[5]. This condition associated with inappropriate feeding practices can be enhanced by the mechanical effects of a nipple or teat[3] which is sometimes left in the infant's mouth for hours[6].

Some researchers have assumed that cows' milk is a cariogenic agent and might induce early childhood caries[7,8]; however, others have reported it as a protective, anticariogenic agent due to its high concentration of Ca and phosphate[9]. Moreover, the buffering activity of proteins such as casein micelles present in this milk[10] might allow the formation of very stable complexes of calcium phosphate[11]. Antibacterial enzymes, vitamin D and fluoride have also been found in cows' milk[12].

There is controversy about the cariogenicity of human milk. Human milk has been related to a sort of caries which is like bottle caries, even though some studies have demonstrated its non-cariogenicity[13]. Others have shown that although it is possible for human milk to cause dental caries, the prevalence would appear to be very low and to be associated with breast-feeding that has continued until at least 2 years of age on many occasions during the day and night[14]. Furthermore, the cariogenic potential of human milk seems not to have been explored experimentally[15] extensively *in vivo*. In a recent study using an animal model, human milk was significantly more cariogenic than cows' milk[16].

Infant formula milk has been the major nutritional supplementation method for feeding babies and children. To manufacture infant formulas, the composition of cows' milk is altered, and fluoridated water is usually added[17]. A high potential for inducing caries could be expected for infant formulas due to their high carbohydrate variability[3]. Thus, it would be wise to add fluoridated water to the formulas in an attempt to reduce their cariogenic effect. The addition of small amounts of fluoride does not affect the appearance, texture and taste properties of this milk[18]. The efficacy of fluoridated bovine milk in preventing dental caries has been studied in Scotland, Hungary, Chile, China, Russia, England and Bulgaria[19,20].

Abbreviations: ppm, parts per million; SDW, sterilised deionised distilled water.

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Milk is widely consumed, especially by children, and thus the interaction between different kinds of milk consumed and caries development should be better understood and investigated. In normal conditions, the salivary flow is reduced during sleep and the contact of saliva with teeth can be restricted by bottle-feeding. Accordingly, even milk, which is considered non-cariogenic, might induce rampant caries[21].

Therefore, the purpose of the present study was to determine the cariogenic potential of infant formula milk reconstituted or not with 10 parts per million (ppm) fluoride compared with cows' and human milk in a desalivated rat cariogenic model, promoting a decrease in the salivary flow similar to that occurring in the mouth of a sleeping infant.

Materials and methods

Treatments

In the present study, sixty female Wistar rats, aged 19 d, SPF (specific pathogen free) from CEMIB/UNICAMP (Brazil) were used. The present study was approved by the Ethical Committee for Animal Research (CEEA/UNICAMP, approval no. 026/1).

The animals were screened for mutans streptococci as previous described[22,23]. The rats were infected with *Streptococcus sobrinus* 6715 at age 20 and 21 d, and received the diet 2000[24] and sterilised deionised distilled water (SDW) with 10 % sucrose *ad libitum* for 7 d to establish infection[23]. Plating of oral swabs revealed that all animals were successfully infected with the *Streptococcus mutans* group.

In an attempt to mimic the situation in infants who suck on the breast or the bottle nipple, the rats were surgically desalivated at age 25 d as previously described[25]. After desalivation, the animals were randomly placed in individual cages and assigned to six experimental groups: group 1, SDW; group 2, 5 % sucrose solution; group 3, human milk (pH 6·8); group 4, cows' whole milk (pH 6·7; 3·85 % fat); group 5, Ninho® (growth supporting, Nestlé do Brasil Ltda., Ituiutaba, Brazil) reconstituted with SDW (this milk was chosen in a first experiment where different brands were evaluated and it showed the most cariogenicity[26]); group 6, Ninho® (growth supporting; Nestlé do Brasil Ltda.) reconstituted with 10 ppm F solution (NaF; Merck S.A., Rio de Janeiro, Brazil). All the animals were subjected to the treatments *ad libitum* but the rats from groups 1 and 2 additionally received a liquid diet (NCP#2; Ross Laboratories, Columbus, OH, USA (for Similac and Promod) and Cargill do Brasil, Sao Paulo, Brasil (Mazola corn oil)) twice per d (5 ml total), by oral administration, to meet their essential nutritional requirements[3,27].

Human milk was obtained from the Clinical Hospital of the Medical School of Ribeirao Preto (Sao Paulo, Brazil) where it was collected and stored under freezing temperature until just before use. Milk was randomly mixed and submitted to gentle sonication twice daily to disperse fat globules.

The cows' milk and infant formulas were purchased from a local supermarket and immediately prepared with SDW according to the manufacturer's instructions. The amount of liquid consumed by each animal was recorded daily.

Animals

At day 21 (experiment completion), the animals were killed by CO_2 asphyxiation followed by decapitation. The half-lower-left jaw of each animal was aseptically dissected, placed in 5 ml of 0·9 % NaCl solution and then sonicated for 30 s (VibraCell 400W; Sonics & Materials Inc., Danbury, CT, USA). The resulting suspension was used to inoculate blood (5 % sheep blood) agar plates (Difco Co., Detroit, MI, USA) using the Whitley Automatic Spiral Platter (DW Scientific, Shipley, West Yorks, England). *Mitis salivarius* agar (Difco Co.) plus streptomycin (Sigma Chemical Co., St Louis, MO, USA) plates were used to determine *Strep. sobrinus* counts[3]. Animal heads were defleshed, and caries scores were assessed by using the Keyes method[28] modified by Larson[29], as follows. Considering unsliced and unstained tooth-smooth surfaces: 'E' for white opaque enamel; 'Ds' for dry and crumbly enamel surface; 'Dm' when dentine is exposed; and 'Dx' when dentine is soft or missing (may be dark in colour). Considering stained, sliced tooth-sulcal surfaces: 'Ds-sulcal' when the pink dye has penetrated into the dento-enamel junction and up to one-third of the depth of the dentine; 'Dm-sulcal' when the pink dye extends more than one-third of the depth of the dentine; 'Dx-sulcal' when the dye has penetrated through the dentine (may be soft or missing).

The carbohydrate concentration in the cows' milk, human milk and infant formula was assessed by the HPLC method[30] by using Waters HPLC Model 480 E (Milford, MA, USA). The total fluoride in all solutions was determined (limit of detection 0·02 ppm F) according to the Taves method[31], by using a specific fluoride ion electrode (model 96-09; Orion Research, Inc., Boston, MA, USA) and a digital ionic analyser (model EA-940; Orion Research, Inc.).

Statistical analysis

The microbial data and caries scores were assessed separately. The results were statistically analysed using the Kruskal–Wallis test (Student–Newman–Keuls as a *post hoc* test) at the significance level of 5 % by using the Bioestat 4.0 statistical software (Bioestat 4.0; Mamiraua Institute, Belem, PA, Brazil).

Results

All animals survived and remained healthy during the 21 d experiment. The animal weight gain varied among the groups because the animals that received nutrition by oral administration gained less weight than the others (Fig. 1). A higher consumption of fluids was observed for groups 4, 5 and 6, without statistical difference among them; however, they were different when compared with the other groups ($P<0·05$) (Fig. 2).

Fig. 3 shows data concerning bacteria counts among all groups tested. The SDW group harboured the highest counts of bacteria ($P<0·05$), while the cows' milk group presented the lowest counts ($P<0·05$). No statistical difference ($P>0·05$) was observed among the other groups (5 % sucrose, and human, Ninho® and 10 ppm F Ninho®). All groups showed a high number or percentage of *Strep. sobrinus* recovered, without statistical difference ($P>0·05$), except for the SDW group ($P<0·05$).

Table 1 shows dental caries scores regarding sulcus and smooth surfaces. The cows', human and 10 ppm F Ninho® milks showed a reduction in total smooth-surface caries scores and did not differ from the SDW group; however, the

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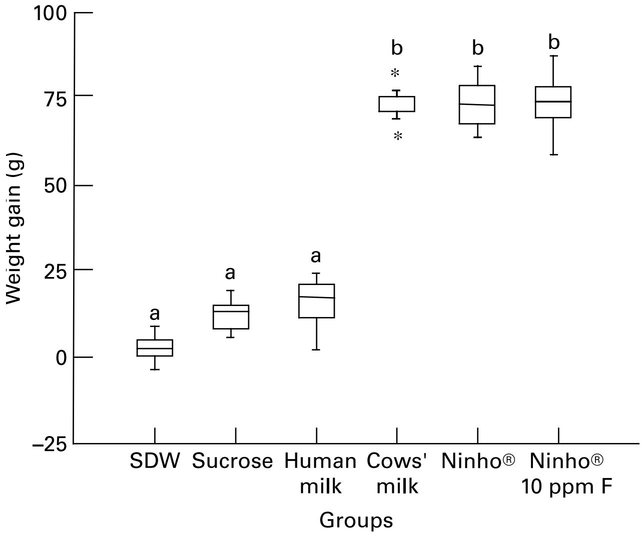

Fig. 1. Weight gain (g) by desalivated animals, after 21 d of experimentation. The central line is the median; the box represents the lower and upper quartiles; the whisker shows the maximum and minimum values; * outliers. SDW, sterile deionised distilled water; ppm, parts per million. [a,b] Median values with unlike letters were significantly different ($P<0{\cdot}05$; Kruskal–Wallis).

cows' milk showed the lowest caries scores ($P<0{\cdot}05$). The total smooth-surface and sulcal caries scores observed for the Ninho® group did not differ statistically from the 5 % sucrose group. The total sulcal caries scores observed for the cows', human and 10 ppm F Ninho® milks were lower than those obtained for the 5 % sucrose and Ninho® groups and higher than that for the SDW group ($P<0{\cdot}05$). The Ds (lesion extended into dentin) and Dm (exposed dentin) severities in smooth-surface and sulcal caries scores basically followed the same profile of the respective total caries scores.

Lactose was the only carbohydrate found in human and cows' milks and the fluoride amount was negligible (Fig. 4). However, Ninho® and 10 ppm F Ninho®, besides glucose

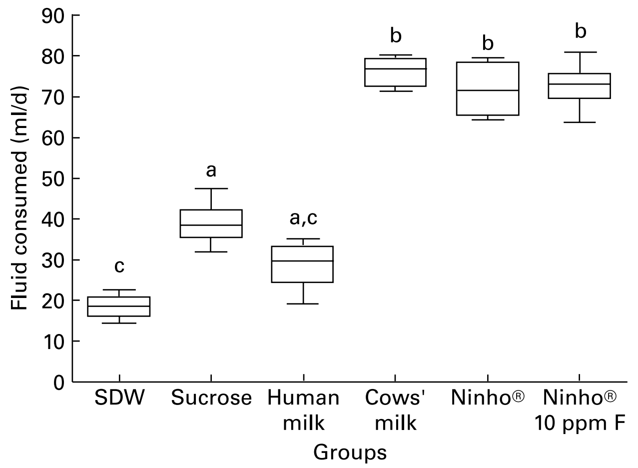

Fig. 2. Amount of fluid consumed (ml) daily by desalivated animals, after 21 d of experimentation. The central line is the median; the box represents the lower and upper quartiles; the whisker shows the maximum and minimum values. SDW, sterile deionised distilled water; ppm, parts per million. [a,b,c] Median values with unlike letters were significantly different ($P<0{\cdot}05$; Kruskal–Wallis).

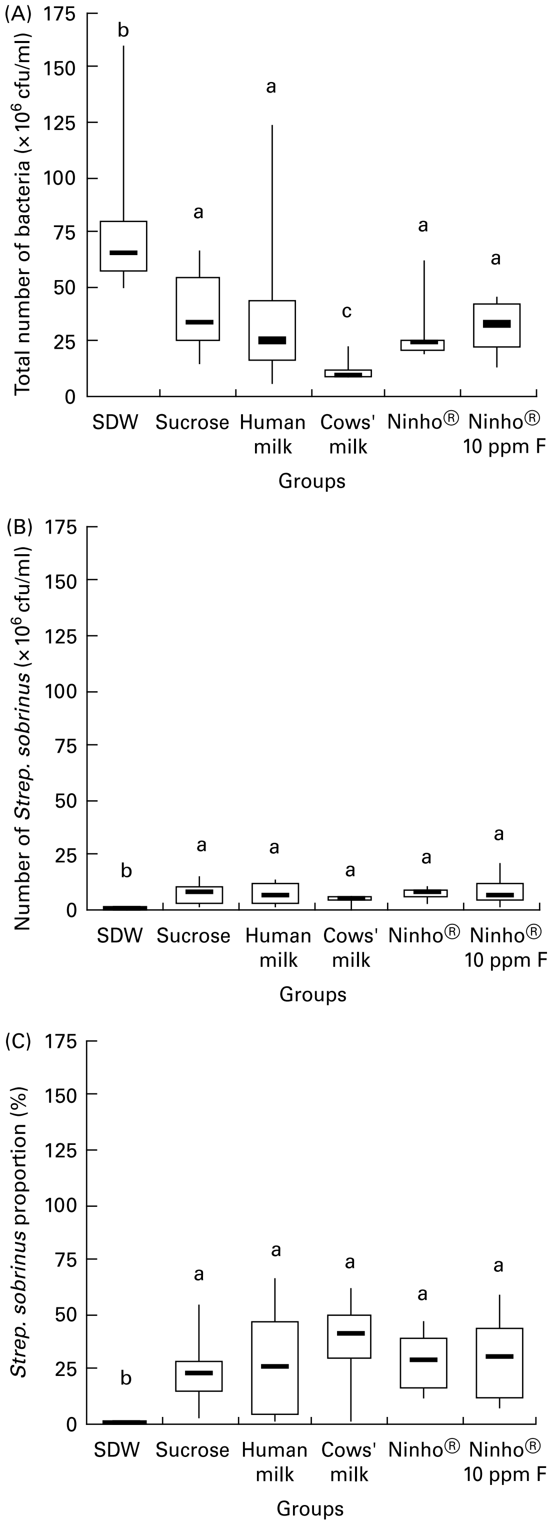

Fig. 3. Total number of bacteria ($\times 10^6$ colony-forming units (cfu)/ml; A), number of *Streptococcus sobrinus* ($\times 10^6$ cfu/ml; B) and *Strep. sobrinus* proportion (%; C) found in desalivated animals, after 21 d of experimentation. The central line is the median; the box represents the lower and upper quartiles; the whisker shows the maximum and minimum values. SDW, sterile deionised distilled water; ppm, parts per million. [a,b,c] Median values with unlike letters were significantly different ($P<0{\cdot}05$; Kruskal–Wallis) considering total number of bacteria, *Strep. sobrinus* number and *Strep. sobrinus* proportion separately.

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Table 1. Total and severity caries scores of smooth-surface and sulcal lesions

(Median values and lower and upper quartiles)

	SDW		5 % Sucrose		Human milk		Cows' milk		Ninho®		Ninho® 10 ppm F	
	Median	Lower–upper quartile	Median	Lower–upper quartile	Median	Lower–upper quartile	Median	Lower–upper quartile	Median	Lower–upper quartile	Median	Lower–upper quartile
Smooth-surface caries												
Total	7·5^{b,c}	3·25–11·75	90·5^{a}	84–106	17^{b}	7·5–26·75	2·5^{c}	1–6·75	56·5^{a}	48·5–59·75	22^{b}	13·5–25·25
Ds	1^{b}	0·25–2·75	62·5^{a}	50·5–74·25	8^{c}	5·25–14·25	1^{b}	0–1	35^{a,c}	31·25–47·75	3·5^{b}	1·25–11·5
Dm	0^{b}	0–0	25·5^{a}	25–37·25	0^{b}	0–0·75	0^{b}	0–1	6·5^{a}	1·75–9·75	0^{b}	0–0
Sulcal caries												
Total	1^{c}	0·25–3·75	72^{a}	69·25–75·75	17·5^{b}	14–25·75	23^{b}	12·25–29·25	66·5^{a}	57·75–68·75	30^{b}	23·5–31
Ds	1^{c}	1–1	35·5^{a}	31–40·5	6^{b}	4·5–9·5	3^{b,c}	2–3·75	24·5^{a}	20·25–35·25	8^{b}	7·25–10
Dm	0^{b}	0–0	22^{a}	20·25–24·5	0^{b}	0–0	1^{b}	0·25–1	12^{a}	11–12	1^{b}	0·25–1·75

SDW, sterile deionised distilled water; ppm, parts per million; Ds, lesion extended into dentin; Dm, exposed dentin.
^{a,b,c} Median values within a row with unlike superscript letters were significantly different ($P<0·05$; Kruskal–Wallis).

and fructose, contained 9·3 % of sucrose and at least 3·5 times more lactose than the other milks; their fluoride concentration was 0·5 and 10·6 ppm, respectively.

Discussion

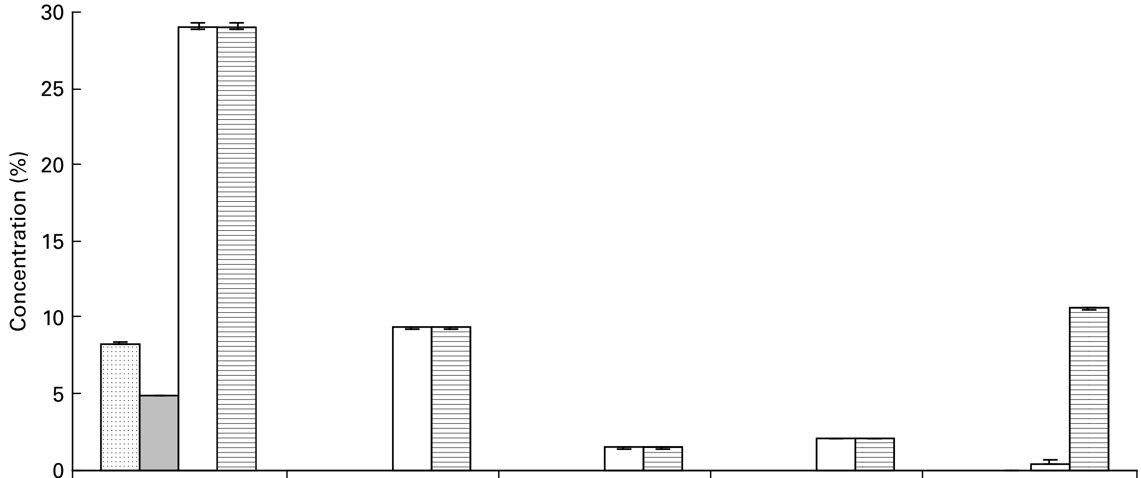
The desalivated rat model, one of the most severe cariogenic challenges available[16], is a very suitable approach for reproducing oral conditions of a milk-bottle-fed infant during sleep, when salivary flow quickly decreases[3].

In the present study, the fluid intake observed for the infant formulas and cows' milk groups was higher than that observed for the controls (5 % sucrose and SDW) and human milk group. This difference might have occurred because the control animals received additional liquid, i.e. an essential nutrition by oral administration twice daily. Although the infant formulas and cows' milk tested showed higher consumption levels when compared with the controls (SDW and 5 % sucrose), they were less cariogenic than 5 % sucrose[10]. Human-milk-fed animals consumed similar amounts to those receiving 5 % sucrose and also showed lower levels of caries, even having the same level of *Strep. sobrinus* infection. It can be assumed that the different amounts consumed did not affect the number of bacteria and consequently caries score. As shown in previous studies[3,27], a weak relationship between the amount of fluid consumed and caries score was also observed in the present study. All the milks and 5 % sucrose maintained the infection of *Strep. sobrinus*, which is in accordance with other reports[3,16]. In addition, 5 % sucrose is not the sole sugar able to maintain the infection; milks containing carbohydrates other than sucrose are more cariogenic than those containing just lactose.

Earlier studies reported good antibacterial activity for some substances and the protein casein found in milk against some oral micro-organisms[11]. Evidence showed that milk composition (Ca, P, peptides) could chemically protect dental enamel against demineralisation[12]. This could also help to explain why 5 % sucrose was more cariogenic than formula milk which contains 9·3 % sucrose. In the present study, cows' milk showed a significant reduction in the number of total microbiota, except for *Strep. sobrinus*. This finding could be related to the antibacterial properties of some substance in cows' milk, such as protein casein[11]. However, Bowen & Pearson[10] using the same animal model could not find any inhibitory effect of bovine milk on total bacteria, including *Strep. sobrinus*.

Milk was in the past reported as being cariogenic[7,8], with a lack of proven evidence, since a distinction has not been drawn between the use of a bottle or nipple *per se* and the fluid content in the feeding bottle[16]. If the bottle contains a caries-promoting substrate, then evidently the potential for damage is greatly enhanced.

Cows' milk is not a caries-promoting substrate, being consumed worldwide, especially by children. It is a natural and healthy beverage containing macro- and micronutrients, which could be beneficial for dental structures[16] and general health. The present results are in accord with those observed in previous studies reporting cows' milk as essentially non-cariogenic[10,16,32]. Natural fluoride was found in the cows' milk tested, but its effect was considered irrelevant due to its low concentration (0·02 ppm F).



	Lactose (%)	Sucrose (%)	Glucose (%)	Fructose (%)	Total fluoride (ppm F)
Human milk	8·3	–	–	–	–
Cows' milk	4·90	–	–	–	0·02
Ninho®	29·00	9·30	1·50	2·10	0·53
Ninho® 10 ppm F	29·00	9·30	1·50	2·10	10·60

Fig. 4. Percentage (w/v) of carbohydrate and total fluoride (parts per million; ppm) present in every milk tested (*n* 10/group). Values are means, with standard deviations represented by vertical bars. (▫), Human milk; (▪), Cows' milk; (□), Ninho®; (▤), Ninho® 10 ppm F; –, no detected sugar or fluoride.

Epidemiological evidence indicates that breast-feeding for over 1 year during the night after tooth eruption might be associated with early childhood caries and with increased prevalence of dental decay[14]. However, other investigations showed no relationship between prevalence of caries and breast-feeding[33]. In the present study, a significantly higher score concerning smooth-surface caries was observed for human-milk-fed animals when compared with cows' milk-fed ones; however, sulcal caries and its severity, concerning human milk, displayed similar scores to those found for cows' milk. Several factors might account for the higher scores of smooth-surface caries observed for the human-milk group. In the present study, human milk was found to contain more lactose (8·3 %) than that (4·9 %) observed for cows' milk. This higher concentration of lactose might reduce dental plaque pH values, but to a lesser extent than might be expected for sucrose. It is also possible that lactose fermentation in cows' milk is slower than that in human milk. Aqueous solutions of lactose have been reported as modestly cariogenic in rat models[32]. It is assumable that other constituents, such as minerals and casein, in cows' milk might overcome the harmful effects of lactose[10].

There is little control over infant formulas regarding their caries potential. In addition, their stagnation inside the mouth due to the reduced salivary flow and the suckle–sleep–suckle cycle might contribute to the enzymic breakdown of their protective proteins, such as casein[34]. The present results, in accordance with previous findings[3], showed cariogenic properties for the infant formula tested, showing a high concentration of sucrose (9·3 %), diversity in carbohydrate, and little amount of fluoride. These characteristics, combined with reduced salivary flow, might result in tooth decay[33].

Other components such as peptides, casein, ions and vitamins have been reported as reducing the cariogenicity of sugar[27]. However, the efficacy of the formula milk compounds in reducing caries was not tested in the present study. The carbohydrate composition in the infant formula tested might be responsible for the differences in caries scores observed between the cows' and human milks. The present results are in accordance with those reported in previous studies using the same desalivated rat model, showing that infant formulas might have potential cariogenic activity[3].

The addition of 10 ppm fluoride to Ninho® milk significantly reduced its cariogenicity, in accord with findings reported in previous studies[19,20]. The fluoride ion added to the formula milk tested showed a remarkable anticariogenic effect, even with one of the most severe cariogenic challenges, such as the desalivated animal model.

While the predominant beneficial effect of fluoride occurs in the oral cavity, its adverse effect, dental fluorosis, might result from fluoride intake during tooth development, or even in later mineralisation stages[35,36]. In places where water is fluoridated, the risk of dental fluorosis development is high[37]. Fluoridated water added to powder milk could result in too high an intake of fluoride if infants are ingesting several doses of reconstituted formula per d[36,37]. Therefore, in areas with non-fluoridated water, milk supplemented with fluoride could help reduce caries prevalence, without risk of dental fluorosis.

The cariogenic potential of any product directly depends on its use. Ideally, children should not be allowed to sleep with a bottle containing any cariogenic substance and dental care professionals should actively discourage their use. Although our findings showed that the addition of fluoride to infant formula milk might significantly decrease its cariogenicity, further studies are needed before implementing the strategy suggested in the present study.

Our data showed that the infant formula milk was as cariogenic as sucrose. Its cariogenic potential was greatly reduced after fluoride addition. Cows' milk has negligible cariogenicity while human milk clearly has some potential for causing caries. The present results call attention to the importance of feeding infants on cows' and human milk, which were observed to be less cariogenic than the artificial milk formulas.

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R. C. R. P. was responsible for the experimental procedures and manuscript writing; L. C. C. assisted in all the experimental procedures; M. C. V. assisted in all the experimental procedures; F. C. G. was responsible for the statistical analysis and graphic creation; J. A. C. was responsible for part of the laboratory analysis; P. L. R. advised, created and designed the study and also assisted in all the experimental procedures.

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