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# Chemical, structural and mechanical characterization of bovine enamel

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ABSTRACT

*Objective:* The purpose of this investigation was to establish microstructure, microhardness, fracture toughness, chemical composition, and crack repair of bovine enamel and to compare these features with their human counterparts.
*Design:* Bovine enamel fragments were prepared and optical microscopy and atomic force microscopy were used to establish microstructure; Raman spectroscopy was used to estimate composition and microindentation using Vickers testing was performed to evaluate hardness.
*Results:* A strong dependence between indentation load and microhardness values was observed, as was the case in human enamel. Similar microstructure and chemical composition between bovine and human enamel, 7.89% lower microhardness and 40% higher fracture toughness values for bovine enamel were found.
*Conclusion:* From a structural and mechanical standpoint, bovine enamel is a suitable alternative to human enamel for *in vitro* testing of dental products.

## 1. Introduction

Human teeth are ideal for *in vitro* studies since they provide an excellent substrate for testing mechanical properties of natural tissues, such as enamel and dentin in different populations, as well as the response of tissues to restorative materials used in dental therapy. However, some limitations are evident when using human teeth, including lack of control of their age, low quantities and sound conditions, and the surfaces are curved and small. Furthermore, ethical issues and infection hazards must be considered (Yassen, Platt, & Hara, 2011). In order to overcome these disadvantages, alternative synthetic (i.e. ceramics, polymethyl methacrylate, etc.) or natural substrates have been used in dental research. Teeth from diverse sources, including swine (Lopes, Markarian, Sendyk, Duarte, & Arana-Chavez, 2006), equine (Edmunds, Whittaker, & Green, 1988), primate (Cox et al., 1998), and bovine (Comar et al., 2012; Isidor, Brondum, & Ravnholt, 1999; Reeves, Fitchie, Hembree, & Puckett, 1995) have been used. Bovine teeth have been the most widely reported in the dental literature due to some advantages, such as ease to obtain in large quantities and sound conditions, have a significantly larger flat surface, and absence of caries lesions that might affect the results (Yassen et al., 2011). Bovine teeth have been used to test dental materials (Ahiropoulos, Helvatjoglu-Antoniades, & Papadogiannis, 2008; Atash & Van den Abbeele, 2005; Nakamichi, Iwaku, & Fusayama, 1983), whitening products (Camargo, Valera, Camargo, Gasparoto Mancini, & Menezes, 2007; de Medeiros, Gonzalez-Lopez, Bolanos-Carmona, Sanchez-Sanchez, & Bolanos-Carmona, 2008; Kwon, Huo, Kim, Kim, & Kim, 2002; Wiegand, Vollmer, Foitzik, Attin, & Attin, 2005), erosion caused by tooth brushing (Kielbassa et al., 2005; Vieira, Lugtenborg, Ruben, & Huysmans, 2006), changes in enamel after fluoride application (Oliveira, Oliveira, Oliveira, Horliana et al., 2018; Oliveira, Oliveira, Oliveira, Sfalcin et al., 2018), demineralization caused by soft drinks (White et al., 2010), among others (Kato, Lancia, Sales-Peres, & Buzalaf, 2010).

The use of non-human teeth in dental research has raised some questions due to compositional and structural differences between human and non-human enamel (Laurance-Young et al., 2011). Teruel, Alcolea, Hernández, and Ruiz (2015) chemically analyzed bovine, porcine, ovine, and human enamel and Ortiz-Ruiz et al. (2018) analyzed these same substrates from a structural perspective finding that bovine enamel is the most similar natural substrate to human enamel. Notwithstanding, many authors have advised extreme care when

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analyzing results from studies carried out on bovine teeth, in general (Fonseca et al., 2008; Ortiz-Ruiz et al., 2018; White et al., 2010), and on bovine dentine (Sanches, Otani, Damiao, & Miyakawa, 2009; Turssi, Messias, Corona, & Serra, 2010). However, several authors recommend using bovine enamel as a substitute to human enamel for *in vitro* assays (Turssi et al., 2010; White et al., 2010).

Some mechanical properties have been evaluated for bovine enamel, including microhardness and fracture toughness. However, these evaluations have been performed on enamel that has been subjected to whitening products or cola drinks (Ameri, Ghavamnasiri, & Abed, 2011; Attin, Muller, Patyk, & Lennon, 2004; Cesar et al., 2009; Park, Kwon, Nam, Kim, & Kim, 2004). Microhardness values between 3.38 and 3.58 GPa using Vickers testing have been reported for exposed bovine enamel (Al-Jobair, 2010; Oskoee, Navimipour, Oskoee, & Moosavi, 2010). Fracture toughness values between 1.0 and 1.5 $MPa*m^{½}$ have been found (Bechtle, Habelitz, Klocke, Fett, & Schneider, 2010). In addition, crack repair has been observed in human enamel (Rivera, Arola, & Ossa, 2013), but no information has been found for sound bovine enamel.

Regarding chemical composition of bovine enamel, more current studies have been carried out to establish the structure of sound bovine enamel (Ortiz-Ruiz et al., 2018; Teruel et al., 2015) and with the purpose of determining compositional changes after application of whitening products (Cesar et al., 2009; Park et al., 2004).

Even though an abundance of papers are available on the changes that bovine enamel undergo as a consequence of the application of diverse dental products, information on the microstructure and mechanical behavior of sound bovine enamel is scarce, so the viability of using this substrate for dental research merits further evaluation. Consequently, the objective of this study was to establish microstructure, microhardness, fracture toughness, chemical composition, and crack repair capability of bovine enamel and to compare such features with their human counterparts.

## 2. Materials and methods

The protocol for enamel cutting, polishing and imaging was as follows: for human enamel, sound, caries-free and without previous restorations third molars extracted for orthodontic reasons were obtained. The teeth belonged to patients from Medellin, Colombia, between 18 and 25 years of age with nearly equal number of males and females. Informed written consent was obtained, following all the protocols required by both Universidad Cooperativa de Colombia (UCC) and Universidad Eafit, to use this biological tissue that would otherwise be discarded. After extraction, teeth were kept in HBSS (Corning, USA) at 2 °C to avoid dehydration and loss of mineral. For bovine enamel, freshly extracted bovine incisors from 36-month-old cattle were obtained from a local slaughterhouse. Teeth were gently cleaned to remove attached soft tissues and were kept in HBBS at 2 °C. Cutting was applied to human enamel following a protocol previously published by our group (Rivera et al., 2013) that was slightly modified for bovine enamel. Briefly, each tooth was sectioned using a diamond disc under continuous water refrigeration to obtain the desired portion of the crown; cusp tips for human teeth and the buccal surface of the incisors for bovine teeth. The direction of enamel rods was previously determined by etching the enamel surface with a solution of 5% phosphoric acid for 5 s followed by washing under running water for 30 s to expose enamel rods. Finally, ten human enamel fragments and eleven bovine enamel fragments were mounted in cold curing epoxy resin and labeled. After 24 h, specimens were polished with silicon carbide papers ranging from 600-grit to 1200 followed by a sequence from 6-μm to 1-μm diamond paste to obtain a mirror-like surface (Polimet I, Buehler, IL, USA). In addition, bovine and human specimens were tested within two weeks of extraction to limit the potential for loss of mineral or organic materials.

In order to assess microstructure for both types of enamel, size and shape, optical microscopy and atomic force microscopy were performed. Optical microscopy (OM) images and micrographs were obtained with an inverted microscope (Axiovert 40 MAT, Carl Zeiss Microscopy, NY, USA) at a magnification of 100x, SEM (Phenom G2, Phenom World BV, Eindhoven, The Netherlands) and AFM images from a 40 μm × 40 μm area (Nanosurf Easyscan 2, Switzerland) were also obtained. AFM micrographs were processed using WSxM software (Horcas, Fernandez, Colchero, Gómez-Herrero, & Baro, 2007). Such images were used to observe enamel rods and determine their direction. All specimens were kept in HBBS throughout the study to avoid dehydration.

For microhardness testing, a load stabilization curve was carried out as previously described by Montoya, Arango-Santander, Pelaez-Vargas, Arola, and Ossa (2015) and 500 g and 10 s of application time were established for bovine enamel. 126 indentations were made (Wilson 401 MVD, Instron Company, MA, USA) following the ASTM C1327 standard (Chen, Chang, Liu, Chuang, & Yang, 2008). As it was previously reported in human enamel by other authors (Giraldez de Luis, Garrido, Gómez-del Rio, Ceballos, & Rodriguez, 2010), hardness is dependent on the load and load stabilization values near 2.00 N have been usually found (Park, Quinn, Romberg, & Arola, 2008; Rivera et al., 2013).

Indentations were made at least two diagonal lengths from the enamel/resin boundary. Vickers hardness was calculated using (1)

$$HV = \frac{0.1891F}{L^2} \tag{1}$$

where $F$ was the applied load and $L$ was the length of the diagonal of the indentation.

Apparent fracture toughness was also calculated since Vickers indentation technique allows creating cracks that extend from the edges of the indentation. 126 measurements were made.

The average crack length was calculated and used to estimate fracture toughness. The following formula was used:

$$K_{c(app)} = 0.0084\left(\frac{E}{HV}\right)^{2/5}\left(\frac{2P}{L}\right)\frac{1}{C^{1/2}} \tag{2}$$

where $E$ is the elastic modulus of enamel, $HV$ is Vickers hardness, $P$ is the load applied, $L$ is the average length of the diagonal, and $c$ is the average length of the cracks. The value of the elastic modulus used was 74 GPa (Giraldez de Luis et al., 2010).

In order to determine crack repair, 33 indentations were selected. Crack length measurements were performed immediately after indentations were made, followed by measurements at 6, 72, and 168 h. Specimens were kept in deionized water at room temperature throughout the experiments. The original crack length ($C_0$) and the subsequent crack lengths ($C_t$) were used to estimate the repaired crack length ($C_h$). The repair efficiency ($n$) was defined as a function of time as follows (Rivera et al., 2013):

$$n = \frac{C_0 - C_t}{C_0} = \frac{C_h}{C_0}. \tag{3}$$

For statistical analysis, the following parameters were calculated: $\mu_1$ (mean global toughness) and $\mu_2$ (mean global hardness). A 95% confidence interval ($\alpha = 0.05$) was estimated for each parameter. The expression of such intervals was given by

$$\mu_i \pm \frac{t_{\left(\frac{\alpha}{2}, n-1\right)} * s}{\sqrt{n}}, \tag{4}$$

where $s$ is the standard deviation. This interval demands that both variables follow a normal distribution. The Shapiro-Wilk test was used to verify that, effectively, both microhardness and fracture toughness followed a normal distribution.

A Raman spectrometer (LabRAM HR, Horiba Jobin Yvon, Kyoto, Japan) was used to determine the chemical composition of human and bovine enamel. Two samples from each substrate were prepared as

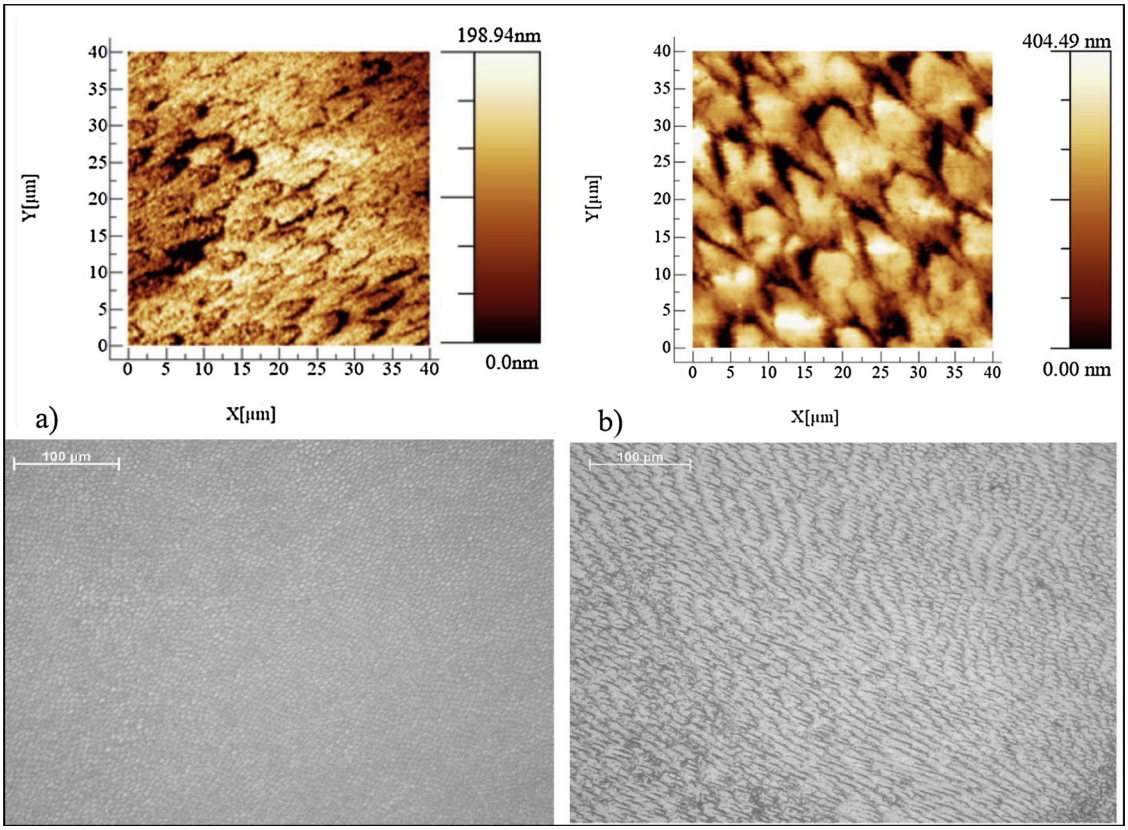


**Fig. 1.** Top: AFM figures of (a) human and (b) bovine enamel. Bovine prisms are larger and show a keyhole shape. Bottom: OM images of (a) human and (b) bovine enamel. Human enamel shows a more homogeneous shape and distribution.

previously described. Measurements were made in the 400–1100 $cm^{-1}$ spectral region. Ten measurements, covering the entire sample surface, were made. Data were normalized and baseline was corrected using data processing software. The peak area associated to the symmetric stretching mode of $PO_4$ at 961 $cm^{-1}$ and $\nu_3$ asymmetric stretching mode of $PO_4$ at 1066 $cm^{-1}$ from hydroxyapatite was examined to analyze the difference in the spectra. An analysis of variance with a 95% confidence level was performed to analyze the differences regarding enamel type

## 3. Results

When comparisons between bovine and human enamel rods distribution and shape were made, bovine enamel rods showed a more flattened shape and were slightly larger (9 µm on average) than their human counterparts (6 µm on average). Fig. 1 shows AFM and OM images of bovine and human enamel.

Bovine enamel exhibited a microhardness value of 3.27 ± 0.2 GPa on average. This value was slightly lower than the value reported for male (3.53 ± 0.16 GPa) and female (3.57 ± 0.11 GPa) human enamel by Rivera et al. (2013). When comparing human and bovine enamel microhardness values, no statistically significant difference was found.

A SEM image from an indentation in topographic mode was obtained (Fig. 2a). In addition, Fig. 2b shows the cracks that radiate away from the impression.

Fracture toughness results of bovine enamel exhibited an average value of 1.04 ± 0.15 $MPa*m^{½}$. This value was 40% higher than the reported for male (0.738 ± 0.02 $MPa*m^{½}$) and female (0.746 ± 0.022 $MPa*m^{½}$) human enamel (Rivera et al., 2013).

Percentage of crack repair over time for bovine enamel is shown in Fig. 2c. All the cracks that were analyzed showed repair, reaching a maximum value of 14% after 168 h.

Fig. 3 shows bovine and human enamel spectra. The spectra for human and bovine enamel show the typical bands observed for hydroxyapatite, in which molecular movements of phosphate groups predominate. The intense peak at 959 $cm^{-1}$ is associated with the phosphate stretching vibration in the mineral apatite component of enamel, the relative area of this peak is associated with the amount of inorganic material in enamel. The 1066 $cm^{-1}$ band is associated with vibrations of carbonate groups and the relative area of the peak is related to the amount of carbonate groups in enamel.

When comparing both spectra, displacements or formation of new bands were not observed. This shows that chemical composition of bovine and human enamel are similar.

The values for areas associated to the bands at 959 $cm^{-1}$ for bovine and human enamel were obtained. When performing intragroup comparisons, no significant differences were found for either band ($p > 0.05$), which shows that the chemical composition of bovine and human samples were similar. The same result was obtained when carrying out intergroup comparisons; therefore, there is no evidence of differences in the inorganic and carbonate contents of both enamels.

## 4. Discussion

The orientation of enamel rods was determined previously to make the indentations perpendicular to them. Bovine crowns were segmented in longitudinal, sagittal, and coronal directions and observed from these angles, as well as from the incisal edge and lingual surface, to determine the orientation of enamel rods. It was found that such rods are perpendicular to the external surface in the middle third of the buccal surface. In addition, the current work found a small difference in the size of bovine enamel prisms, being more flattened and slightly larger than human prisms (Rivera et al., 2013). In accordance with the work of Pizarro Sanchez, Otani, Damião, and Miyakawa (2009), bovine prisms showed a similar size (~8 µm) and a keyhole shape under AFM

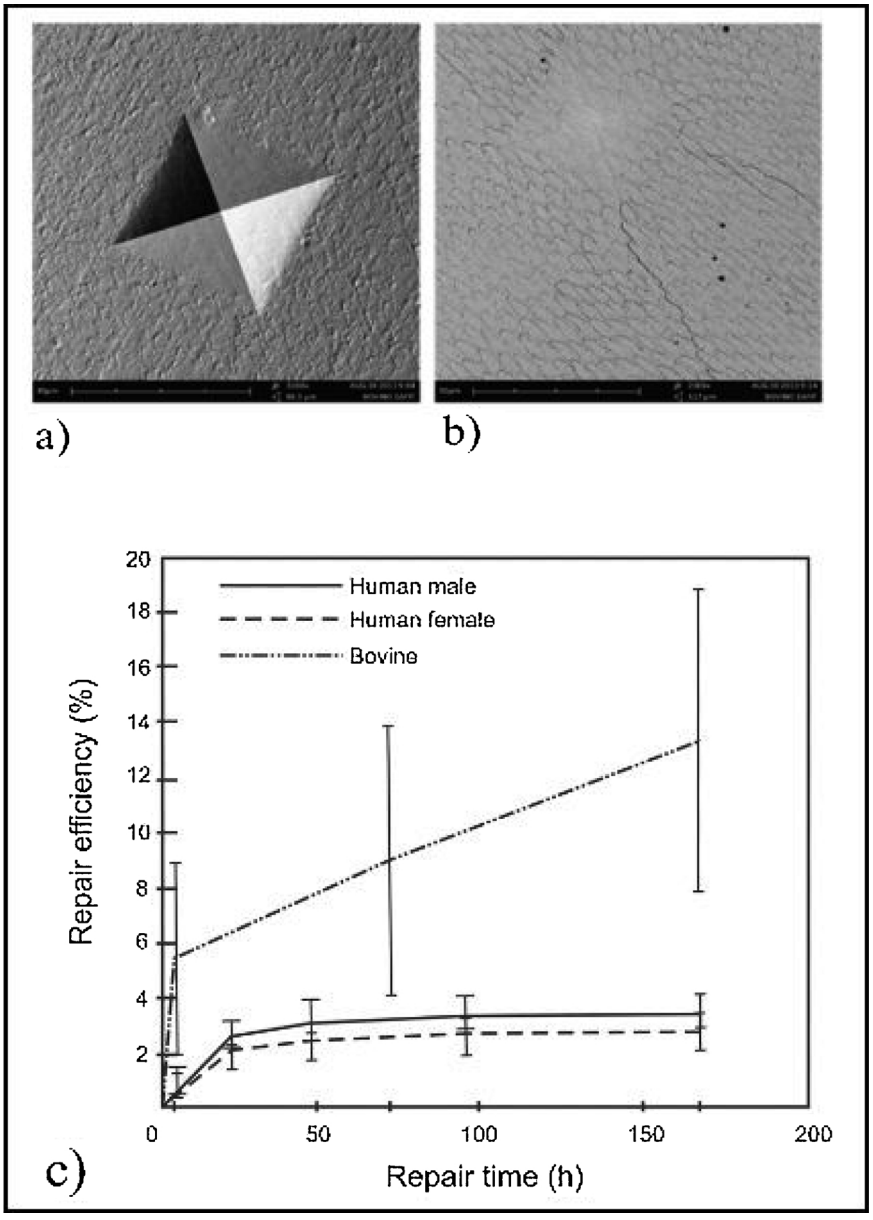


**Fig. 2.** Crack growth and repair of bovine enamel. a) Indentation in topographic mode; b) cracks arising from edges of indentation; c) % increase in crack repair over time for human and bovine external enamel (human data in Fig. 2c is published in Rivera et al. (2013) and used with permission from the authors).

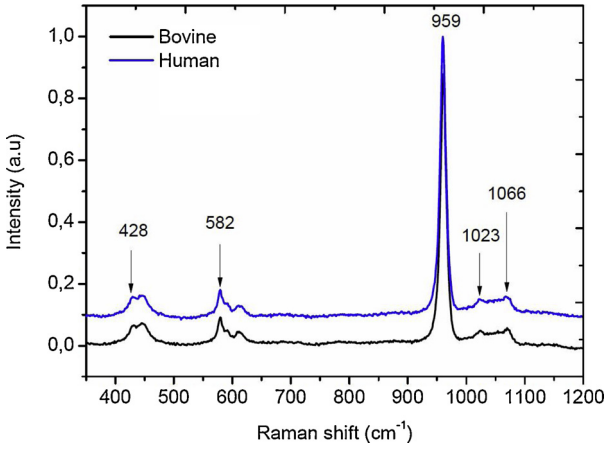


**Fig. 3.** Raman spectra of bovine and human enamel shows similar composition. No displacements or formation of new bands were observed.

scanning, which was consistent with the findings of this work.

The effect of the indentation load on microhardness was established. For low indentation loads (10 g), enamel microhardness reached values around 6 GPa. According to results from human enamel, this high value may be considered as the hardness of one enamel rod (Rivera et al., 2013). For indentation loads after 500 g, a plateau value around 3 GPa was obtained, which is more indicative of the overall enamel hardness.

Investigations on mechanical properties, such as microhardness and fracture toughness, of sound bovine enamel are scarce. However, different investigations to examine the changes caused on bovine enamel after subjecting it to different products, such as whitening substances or acidic solutions, are found in the literature. As such, Fernández, Abbiati, Cabrera, and Martínez (2011) carried out a study on enamel microhardness of two different cattle breeds. The methodology of their study differs from the methodology used in the present work since they measured enamel microhardness at three different points on the incisal edge, while microhardness was measured on the buccal surface at the middle third of the crown in the present investigation according to the

aforementioned explanation. Such difference in the methodological approach may explain the difference in enamel microhardness found in both investigations. In an investigation carried out by Oskoee et al. (2010) to determine the changes in enamel microhardness after treatment with 10% sodium ascorbate, the values reported for sound bovine enamel microhardness were similar to the data found in the present work. Zanet, Fava, and Alves (2011) used a different methodology to establish bovine enamel microhardness changes before and after subjecting the enamel to whitening products and acidic solutions. Their microhardness values were lower than the results from our work. However, the indentation load they applied on the enamel surface was much lower than the load applied in this work. Park et al. (2004) evaluated the changes in enamel microhardness after exposing it to 30% hydrogen peroxide. Even though they used a different methodological approach, enamel microhardness of the control group (~3.1 GPA in distilled water) was similar to the findings of the present investigation. Ameri et al. (2011) evaluated the fracture toughness of bovine enamel at different time intervals when subjected to a whitening agent and found a reduction in fracture toughness as the enamel is exposed to such agent, although the difference was not statistically significant. The values reported by these authors are higher than the values found in the present investigation.

For the purpose of comparing human and bovine enamel microhardness and fracture toughness, data were extracted from Rivera et al. (2013). According to the results of such study, there is no statistically significant difference in female and male human enamel. Based on the values found in the present work, human enamel showed slightly greater microhardness than bovine enamel, but such difference was not statistically significant. This finding may be explained by the fact that cattle diet consists predominantly on grass (fresh or conserved) and concentrate (Warren et al., 2008), whereas human diet is much more diverse and includes harder foods like seeds or nuts (Constantino, Wood, & Lawn, 2008). Mastication of such hard foods will require that human enamel be harder to withstand the higher forces that will be imposed on this tissue. On the other hand, fracture toughness was higher for bovine enamel and the difference was statistically significant. It is important to indicate that in the work of Rivera et al. (2013), fracture toughness was measured at several depths from the surface. In the current work, fracture toughness was measured only at the surface, so the increase in this value as the depth of measurement increases reported by these authors could not be corroborated in the present investigation. Such lower hardness and higher fracture toughness of bovine enamel might be explained by higher protein content in the former due to the age of bovine teeth (36 months) which might also explain the higher crack repair capability displayed by bovine enamel since protein content is responsible for the viscoelastic component. A 14% crack repair was observed after 168 h. Results for human enamel (Rivera et al., 2013) showed lower repair (4%) at the external surface after 168 h, but increased to 10% near the dentin-enamel junction (data not shown). According to Rivera et al. (2013), this reduction in crack length with time could be the result of viscoelastic recovery and/or via the operation of crack closure stresses. In addition, these authors demonstrated evidence of organic proteins bridging the crack, which assists in the crack closure process.

Raman spectroscopy showed no major differences in the chemical composition of human and bovine enamel, even though the average age of human and bovine subjects was different.

## 5. Conclusions

Bovine enamel shows similar hardness, higher fracture toughness, and higher crack repair capability than human enamel. The chemical composition of both types of enamel is similar. Therefore, bovine enamel is a suitable alternative to human enamel for *in vitro* testing of dental biomaterials from mechanical and chemical perspectives.

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## Declaration of Competing Interest

None.

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