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In Vitro Evaluation of Bond Strength of Adhesive Systems to Dentin and Stability of the Hybrid Layer

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ABSTRACT

Objective: Contemporary dental adhesives demonstrate strong durable adhesion to enamel while long-term bonding stability to dentin remains uncertain. This in vitro study aimed to compare conventional dentin conditioning protocols supplemented with protease inhibition and chelate-based conditioning protocols.

Materials and Methods: Four groups of extracted teeth were subjected to four different protocols. Groups A and B were conditioned as conventional etching techniques: in group A orthophosphoric acid 37%, chlorhexidine 0.20%, and adhesive, group B self-etch two-step adhesive and chlorhexidine 0.20%, followed by a composite layer. Groups C and D were treated using extrafibrillar demineralization techniques: Group C ethylenediaminetetraacetic acid (EDTA) (17%) and a self-etch two-step adhesive process, and Group D polyacrylic acid salt solution (225 kDa) and adhesive, followed by a composite layer. Shear bond strength tests, scanning electron microscopy evaluations, and zymography were conducted to evaluate the adhesion forces, stability and proteolytic activity of the hybrid layer before and after accelerated aging. Data were analyzed with two-way ANOVA and Tukey tests for SBS analysis. Non-parametric Mann–Whitney test and an Aligned Rank Transformation model were performed for the zymography analysis. The Weibull distribution test assessed each type of failure.

Results: No statistically significant differences in bond strength were observed immediately after adhesion among groups ($p \geq 0.05$). After aging, significant differences appeared ($p < 0.05$). Groups A and D showed stability in bond strength. Proteolytic activity differed significantly between conventional and extrafibrillar groups both immediately and after aging ($p < 0.05$). Group D showed no significant changes pre- or post-aging ($p > 0.05$). Group C had higher stability than groups A and B.

Conclusions: Compared with conventional conditioning protocols combined with protease inhibition, the evaluated chelate-and-rinse protocols showed improved maintenance of bond strength and reduced proteolytic activity over time under the conditions of this in vitro study.

Clinical Significance: The application of a novel extrafibrillar demineralization method represents a significant improvement in dentin bonding protocols, as it preserves collagen structural integrity and reduces proteolytic activity. This results in a more stable adhesive interface over time, with no loss of bond strength after aging, potentially increasing the longevity of restorations. Clinically, this strategy may enhance the predictability and durability of adhesive procedures. In the context of esthetic dentistry, improved bond stability contributes to better marginal integrity, reduced risk of discoloration and microleakage, and longer-lasting esthetic outcomes, ultimately leading to more reliable and visually stable restorations over time and patient comfort.

1 | Introduction

The immediate adhesion of contemporary adhesives to tooth structure appears to be favorable, especially when the bonding substrate is enamel. However, the durability of the adhesion to dentin remains questionable over time due to the degradation of the hybrid layer [1]. This degradation is produced by the hydrolysis of the polymerized hydrophilic resin and the activity of the Metalloproteinases (MMPs) and Cathepsines (CTs) [1, 2] which are activated and found in the demineralized collagen of the hybrid layer [3–5] The clinical manifestations of the aforementioned degradation include the loss of retention and adaptation, dentin hypersensitivity, marginal pigmentation, secondary caries and loss of restoration (Figure 1). The "wet bonding" technique, alternatively termed the "modified wet bonding" technique, has been posited as a substitute for conventional procedures. While these techniques have been shown to facilitate immediate adhesion to the substrate, their ability to ensure long-term stability of the adhesive interface remains uncertain [6–8]. The utilization of self-etch adhesives with elevated concentrations of 10-methacryloyloxydecyl dihydrogen phosphate (10 MDP) [9, 10] and non-specific synthetic inhibitors, such as chlorhexidine and collagen cross-linking agents, has been a subject of investigation. The use of chlorhexidine has shown efficacy in the inactivation of MMPs and CTs, but the effect appears to be limited over time [11–13]. The electrostatic nature of chlorhexidine–dentin binding makes it water-soluble and unstable over time. The efficacy of collagen cross-linking agents, flavonoid compounds, and polyphenols, such as proanthocyanidins, quercetins, and curcumins in counteracting MMP activity has been demonstrated. However, their clinical application is constrained by their complexity and protracted operational duration [14]. Glutaraldehyde, a well-established cross-linking agent, has demonstrated efficacy in vitro; however, its clinical utilization is hindered by its reported toxicity [15, 16].

A recently proposed approach, the *Chelate and rinse* technique [17] aims to selectively demineralize the extrafibrillar matrix of dentin collagen with high molecular weight chelating agents. This is based on the theory that these substances cannot penetrate the intrafibrillar structure of dentin collagen, and prevent collapse while keeping the collagen spacing and network intact. This approach may enhance the durability of the interface and prevent the activation of proteases. Studies have utilized various chelating substances, including ethylenediaminetetraacetic acid (EDTA), glycol-chitosan, and polymeric chelators, such as the sodium salt of polyacrylic acid (PAANs). The outcomes of these studies are encouraging, as the substances appear to possess the capacity to demineralize the dentin surface while preserving the intrafibrillar structure of collagen. Additionally, they demonstrate anti-collagenolytic activity and maintain substrate-resin adhesive forces in a manner analogous to conventional techniques employing self-etching agents. However, it is imperative to note that further research and long-term studies are necessary to fully elucidate the potential of these substances [17–23].

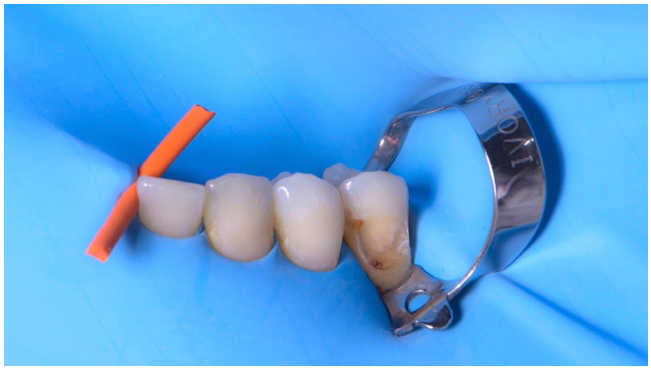

FIGURE 1 | Representative image of the restorative procedure performed for a previous failed restoration with exposed dentin substrates under rubber dam isolation.

Other studies have combined various conventional dentin conditioning techniques, such as total etching or self-etching, with MMP inhibitors including chlorhexidine, EDTA, or benzalkonium chloride. These studies have demonstrated that the combination of these techniques can positively influence adhesive durability and delay the appearance of proteases [24, 25].

Unlike chelate-based conditioning strategies, both phosphoric acid etch-and-rinse and self-etch approaches are generally regarded as non-selective demineralization strategies, as their low-molecular-weight acidic species can diffuse into and dissolve mineral phases from both extra- and intrafibrillar regions.

However, to the authors' knowledge, there are no previous studies comparing conventional conditioning techniques with MMPs and CTs inhibitors and the "chelate and rinse" technique.

The present study aims to assess the bond strength (SBS) and stability of the adhesive interface, analyzed by scanning electron microscopy (SEM) images and zymography, comparing different dentin conditioning techniques. Within the scope of the study, a comparative analysis was undertaken between non-selective/extrafibrillar demineralization groups and exclusive extrafibrillar demineralization techniques. Based on the existing evidence, it was hypothesized that different experimental conditions would result in significant differences in adhesion forces and in the morphology and metalloproteinase activity of the adhesive interface, both before and after aging.

2 | Material and Methods

2.1 | Preparation of Specimens and Pretreatment Protocols

The sample size was calculated using G*Power software (version 3.1.9.7, Universität Düsseldorf, Germany), with an alpha level of 0.05, a beta level of 0.20, and an estimated effect size of $d = 0.80$. A total of 56 teeth taken from sound molars and premolars extracted for medical, orthodontic or periodontal reasons were used for the study. Teeth were excluded from the study if they exhibited any of the following characteristics: root canal treatments, caries, enamel or dentin structural

defects and cracks and/or fissures. Following extraction, the specimens were stored in a solution of NaCl 0.7% and sodium acid 0.02% at 4°C for a maximum of one week [18] This was done to clean and remove any organic residues that may have remained adhered to the tooth surface following extraction. The calculus was then removed using an ultrasound device (Satelec Newtron P5XS), and the stains were eliminated through the application of polishing brushes and pastes (Enhance Dentsply). Following this, the specimens were stored in deionized water. Each specimen was then placed into a self-curing acrylic resin base (SamplKwick Powder Fast Cure Acrylic 20-3562, Buehler), with the root portion of each specimen being kept inside the acrylic base to facilitate handling. The coronal part of the specimens was then removed using a trimmer (Renfert) diamond blade machine under irrigation, thus exposing the mid dentin substrate and preparing the specimens for conditioning (Figure 1). The surface of the samples was then polished with a 600-grit silicon carbide wet paper (CarbiMet PSA; Buehler) for 30 s to create a standardized smear layer. The samples were randomly divided into four equally sized groups of 14 samples ($N = 14$) using a simple randomization system with balanced distribution with R software.

The dentin surface of the specimens was then subjected to treatment in accordance with the four distinct protocols that had been studied:

- Group A (control group) ($n = 14$): The teeth were etched with orthophosphoric acid (ScothBond Etchant, 3M) for 15 s, rinsed with water for 30 s, and chlorhexidine 0.2% was rubbed on the surface with a cotton pellet for 60 s. Following this, a two-bottle/two-step adhesive (OptiBond FL, Kerr) was applied following the manufacturer's instructions (Figure 2).
- Group B ($n = 14$): The two-bottle/two-step self-etch adhesive (Clearfil SE Bond 2, Kuraray) was applied on the dentin: between the acidic primer applied for 20 s and the bonding, chlorhexidine 0.2% was rubbed on the surface with a cotton pellet for 60 s (Figure 2).
- Group C ($n = 14$): EDTA 17% (Canalpro EDTA 17%, Coltene) was applied to the dentin surface for 120 s, after which a two-bottle/two-step self-etch adhesive (Clearfil SE Bond 2, Kuraray) was applied in accordance with the manufacturer's instructions (Figure 2).
- Group D ($n = 14$): 60-225 kDa high molecular weight polyacrylic acid sodium salt (Poly(acrylic) acid solution, Polyscience) was applied for 120 s on the surface and rinsed, then two-bottle/two-steps adhesive (OptiBond FL, Kerr) was applied following the manufacturer's instructions (Figure 2).

A 4 mm layer of microhybrid composite (Filtek Supreme, 3 M) was applied on the surface of the four groups in two increments and polymerized with a LED light curing device (3 M Paradigm DeepCure LED Curing Light) (Figure 2).

The specimens were then stored in deionized water at 37°C for 24 h. After this, the samples were cut in half with a precision cutting machine (Micromet M, Remet), leaving a semicircle on each sample with a standard surface area of 3 mm^2.

2.2 | Aging

The samples were subjected to accelerated aging in a cold-heat thermocycling machine. They were subjected to a total of 5000 cycles with an immersion time of 20 s at a temperature of 5° ±5 and 55° ±5 in an artificial saliva solution, which was made using the Fusayama–Meyer formula with a neutral pH equivalent to 7 to simulate 6 months of intraoral life [26–28].

2.3 | Interfacial Evaluation

The samples were sequentially wet polished using a semi-automatic polisher (BUEHLER 250 MT) with diamond cloths of 74 μm for 10 min, −9 for 25 min, −3 for 40 min, and −1 for 10 min. Following the polishing procedure, the samples were subjected to drying for a period of 24 h within an oven (Carbolite PF120) maintained at a temperature of 60°C.

Scanning electron microscopy (SEM Phenom TM G2 Desktop) was utilized to analyze the interface of the different groups before and after aging. SEM images were captured from various areas of the samples following a predefined and standardized imaging protocol [29]. Two specimens per group were analyzed immediately and two additional specimens per group after accelerated aging.

2.4 | In Situ Zymography

The in situ zymography technique is used to detect the proteolytic activity of MMPs and CTs following an adhesive procedure. A fluorescent in situ zymography assay was used to detect MMPs activity at the adhesive-tooth interface by confocal microscopy. The adhesive-tooth surfaces were directly incubated onto 200 μL of a gelatin-FITC substrate for MMPs (EnzChek, DQ Gelatin From Pig Skin, Fluorescein Conjugate) in a humidified chamber (Heraeus, Hanau, Germany) at 37°C. After 48 h samples were imaged in a laser scanning confocal microscope (FV1200, Olympus at the Cytometry and Fluorescence Microscopy) with a 60× WImm objective. Z-stack images were obtained under the following scanning conditions: ex/em; 488 nm/500–530 nm; XY scan resolution: 1024 × 1024; Z-stacks: 0.5 um step; 11–20 um depth. Three regions of uniform dimensions, located at the midpoint between the two enamel-dentin junctions, were captured to assess gelatinolytic activity. Microscopy was conducted by an independent evaluator who was blinded to the treatment applied to the tooth segments. Hydrolysis of gelatin conjugated with quenched fluorescein is indicative of endogenous gelatinolytic activity and was expressed as a percentage of the green fluorescence emitted within the HL. The results were then quantified using Fiji ImageJ interpretation software. Two samples from each group were immediately analyzed under zymography, while two additional samples were subjected to a thermocycling process for accelerated aging [21].

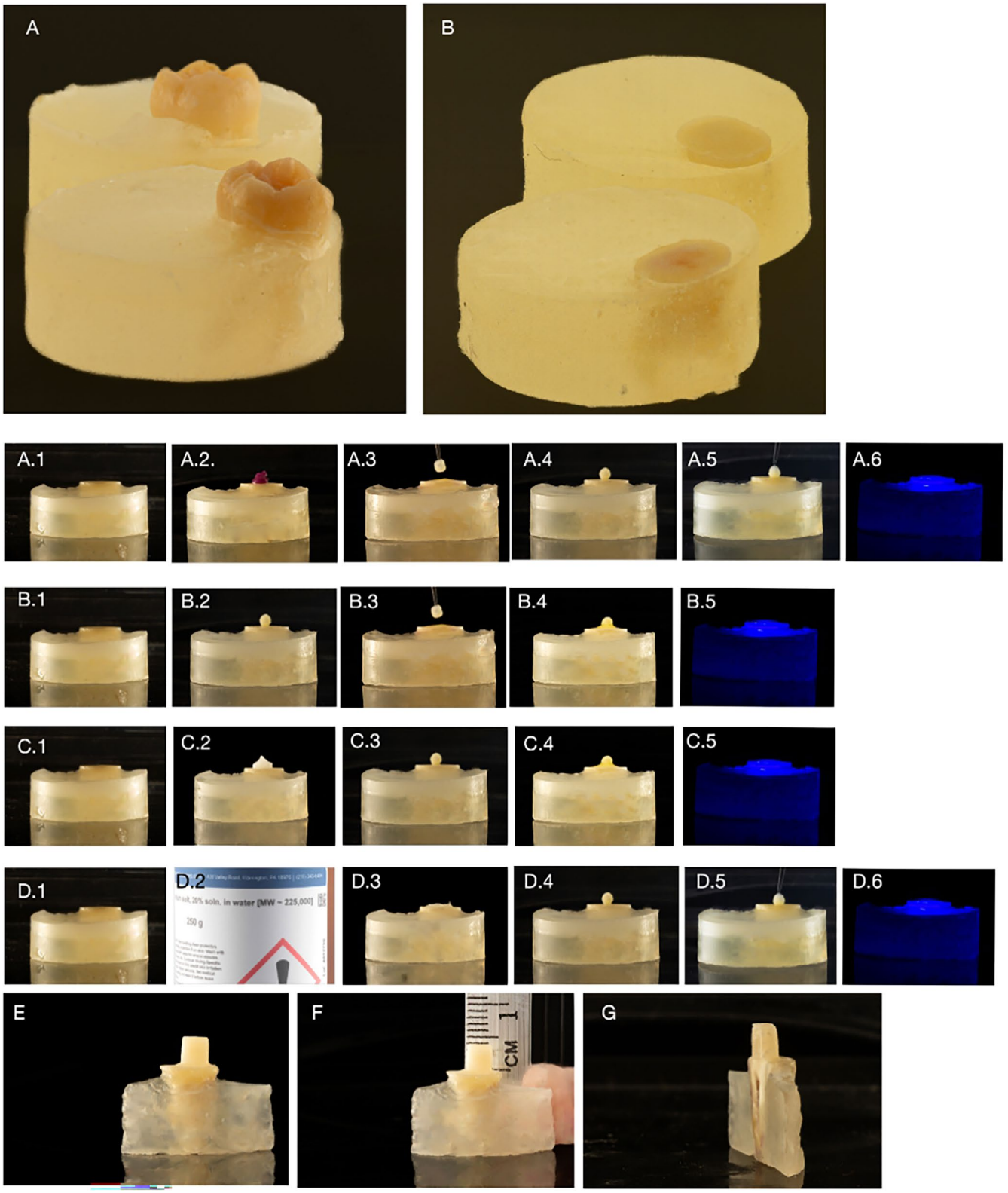

FIGURE 2 | (A) Specimens placed in the resin base. (B) Specimens with the coronal part trimmed and the mid dentin exposed. Pretreatment protocol for group A. (A 1–6). (A.1) Polished surface, (A.2) orthophosphoric acid 37%, (A.3) clorhexidine 0.20%, (A.4) primer, (A.5)bonding, (A.6) light curing. Pretreatment protocol for group B (B 1–5). (B.1) Polished surface, (B.2) acidic primer, (B.3) clorhexidine 0.20%, (B.4) bonding (B.5) light curing. Pretreatment protocol for group C (C 1–5). (C.1) Polished surface, (C.2) EDTA 17% 120 s, (C.3) acidic primer, (C.4) bonding (C.5) light curing. Pretreatment protocol for group D (D 1–6). (D.1) Polished surface, (D.2) PAAN solution 225 MW, (D.3) PAAN 120 s dentin treatment, (D.4) primer, (D.5) bonding, (D.6) light curing. (E, F) 4 mm composite button, (G) specimen cut in 1/2 with precision cutting machine.

2.5 | Shear Bond Strength (SBS) Test

The study was conducted in accordance with the ISO 29022 [30], additional aspects related to tooth substrate preparation and adhesion testing strategy were considered according to ISO/TS 4640 [31].

Five samples from each group ($n = 5$) were immediately subjected to SBS testing, while another five ($n = 5$) were subjected to an accelerated aging test equivalent to 6 months in vivo conditions.

The samples were held in the clamps and subjected to shear force in a universal testing machine (AG-X Series, Shimadzu) at 0.5 mm/s crosshead speed. The values were computed (MPa) by dividing the maximum load force by the adhesion surface.

2.6 | Failure Mode Analysis

Optical stereo microscope (Leica MZ6) was used to analyze the failure mode. The failure modes were categorized into: adhesive failure (A), cohesive fracture (C), and mixed failure (M).

2.7 | Statistical Analysis

The statistical analysis was performed using R (version 4.4.1) within the RStudio environment (version 2024.09.0) R Foundation for Statistical Computing, Vienna, Austria.

For all variables, the predefined statistical significance was $\alpha = 0.05$.

2.7.1 | Zymography Statistical Analysis

After checking for normality with Kolmogorov and Shapiro–Wilk test ($p < 0.05$) a non-parametric Mann–Whitney and an ART (Aligned Rank Transformation) model were performed for the zymography analysis, with the objective of studying if there were significant differences in the means of the variable %Area for the independent qualitative variables groups (A, B, C, D), pre/post-aging conditions, and the interaction between them.

2.7.2 | SBS Statistical Analysis

A two-way ANOVA was conducted to assess the presence of significant differences in the mean values of the MPa variable, considering the independent qualitative factors: groups A, B, C, D, pre−/post-aging conditions, and their interaction between them.

Tukey HSD multiple comparison analysis was conducted to identify which groups and interactions were significantly different in the study.

Chi-squared and Fisher's exact tests were conducted to ascertain the existence of a significant relationship between the qualitative variables.

2.7.3 | Failure Mode Statistical Analysis

The Weibull distribution test was used to assess each type of failure (adhesive failure (A), cohesive failure (C), and mixed failure (M)). This analysis enabled the determination of the shape (β) and scale (η) parameters, which describe the behavior of strength according to this distribution.

3 | Results

3.1 | SEM Images

The SEM images illustrate the dentin-adhesive-composite interphase in groups A, B, C, and D prior to aging. Subsequent to the aging process, alterations in the form of voids or changes in appearance are observed within these interphases in groups A and B. In contrast, groups C and D exhibited stability or no changes, respectively (Figures 3 and 4).

3.2 | Zymography Analysis

The results of the zymography analysis are presented in Figure 5, which illustrates the percentage of emitted fluorescence for the four groups. A high level of significance was observed in the immediate adhesion phase, dependent on the treatment applied. Group A, followed by Group B (conventional etching), exhibited the highest fluorescence levels, while Groups D and C (extrafibrillar demineralization) demonstrated the lowest values. The difference was not statistically significant between the conventional etched groups (A vs. B, $p = 0.183$) or the extrafibrillar conditioned groups (C vs. D, $p = 0.10$). However, a significant difference was observed between the conventional and extrafibrillar categories ($p < 0.0001$). This difference was qualitatively observed by the intense green fluorescence emitted within the hybrid layer, being higher in groups A and B and barely non-existent in the groups C and D (Figure 6).

Following aging, a significant overall effect on fluorescence was identified ($p < 0.0001$). Group B exhibited the highest fluorescence levels, followed by Group A, and then Groups C and D. The difference remained non-significant within the conventional (A vs. B, $p = 0.239$) and extrafibrillar (C vs. D, $p = 0.183$) groups, but highly significant between them ($p < 0.0001$). Group D exhibited the most stability after aging, with no significant pre-post changes ($p = 0.092$). In contrast, significant changes were observed for Group A ($p = 0.016$), Group B ($p < 0.0001$), and Group C ($p < 0.0001$). A detailed analysis of cross-effects is provided in Table 1. These trends can be visually corroborated by the fluorescence intensity patterns observed under microscopy (Figure 6).

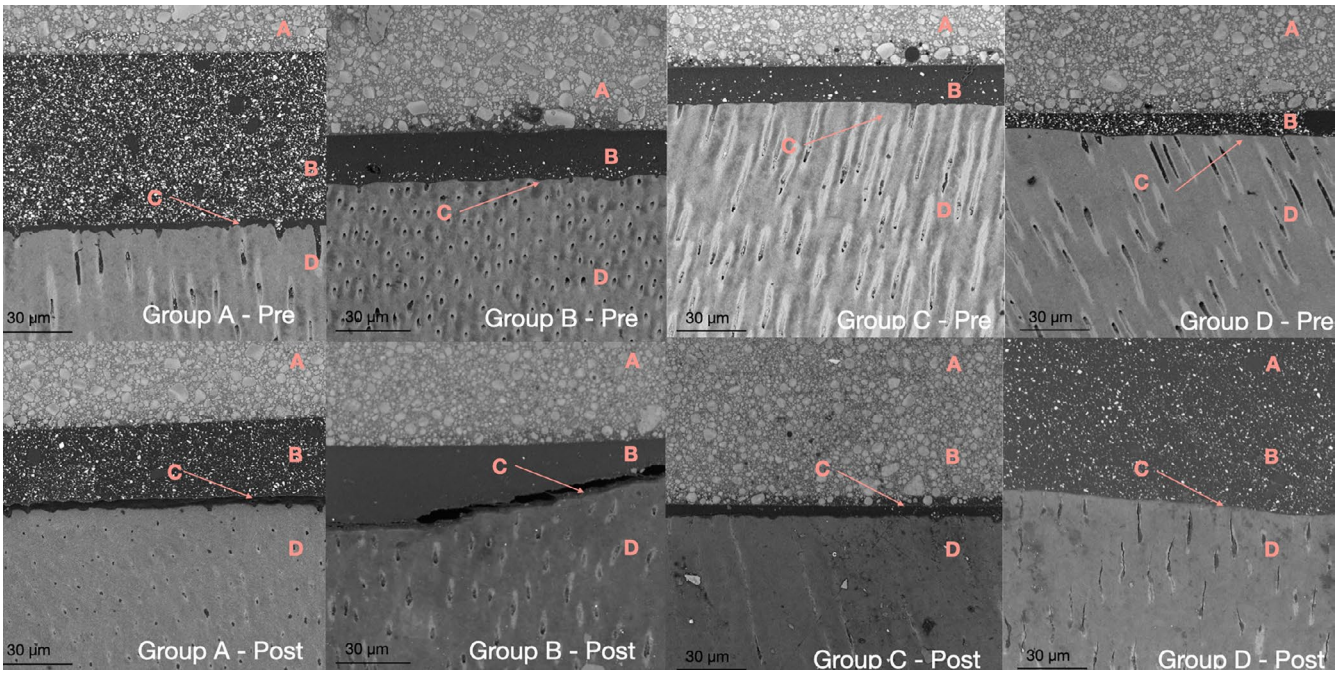

FIGURE 3 | Representative SEM images of groups A, B, C, and D of the adhesive interphase before and after aging. In all images A shows composite layer, B adhesive layer, C adhesive interface, and D dentin substrate. After aging, alterations in the form of voids or changes in appearance are observed within these interphases in groups A and B. In contrast, groups C and D exhibited stability or no changes, respectively.

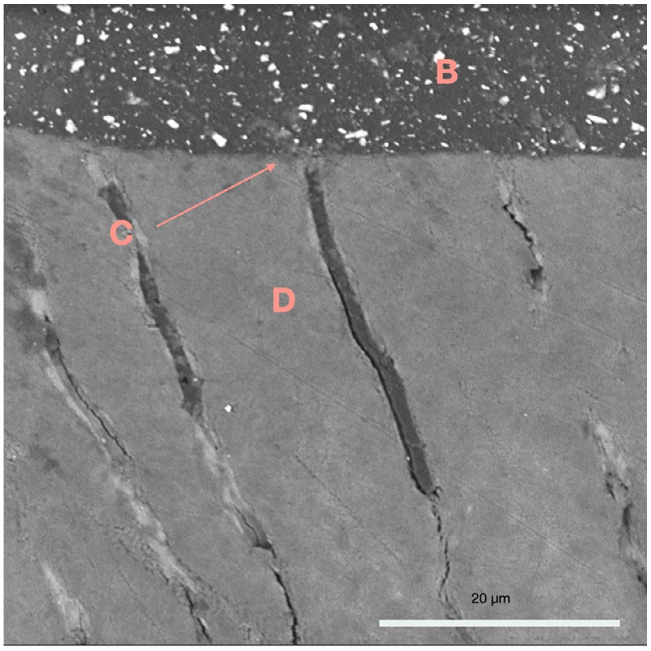

FIGURE 4 | Representative SEM image of group D after aging which exhibited stability of the adhesive interphase. B shows adhesive layer, C adhesive interface, D dentin substrate.

3.3 | SBS

The SBS computed for the four groups is plotted in Figure 7. The quantitative data (mean, standard deviation, median, quartiles, minimum, and maximum) for each group pre- and post-aging are presented in Table 2. Among the analyzed groups, no significant differences were detected in immediate adhesion forces. However, subsequent to the aging of the samples, a two-way ANOVA test revealed significant differences between immediate adhesion forces and those after aging ($p < 0.001$). A more detailed analysis of the cross-effects between treatments was then conducted; the results of this analysis, including both the ANOVA and Tukey HSD tables showing effects between groups pre- and post-aging as well as cross-effects, are presented in Table 3.

The findings revealed that, subsequent to the aging process, there were substantial variations in adhesion forces among the treatment groups. The group that exhibited the most optimal adhesion outcomes was group D (PAANs 220 kDa, 120 s), followed by group C (EDTA 17%, 120 s + two-step self-etch adhesive), group B (two-step self-etch adhesive + chlorhexidine 0.2%), and finally group A (phosphoric acid 37%, 15 s + chlorhexidine 0.2% + two-step adhesive).

Statistically significant differences after aging were identified between groups A and D ($p = 0.0044$), between groups B and D ($p = 0.0006$) and C and D ($p = 0.034$). For the remaining comparisons (C-A, B-A, C-B), none were statistically significant ($p > 0.05$), indicating an absence of substantial differences. Group D exhibited the most stability in adhesion forces following the aging process, with no statistically significant alterations observed pre-post-aging, also group A. In contrast, significant changes were observed in the other groups: group B ($p = 0.00057$), and group C ($p = 0.015$).

3.4 | Failure Mode Analysis

The probability of the different types of fracture depending on the achieved strength according to the Weibull analysis test are in Figure 7, which show that the value of $\beta = 3.37$ suggested that

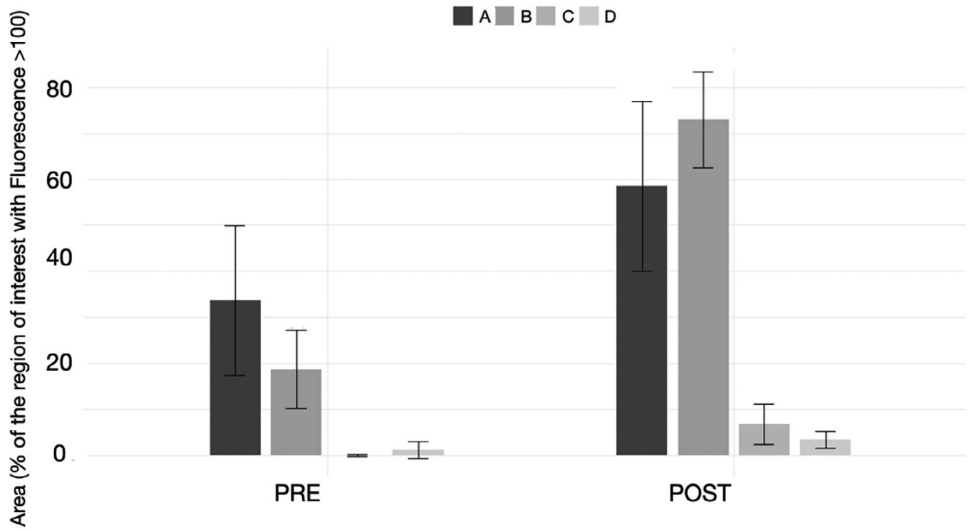

FIGURE 5 | Zymography values of each group, means and standard deviation before and after aging.

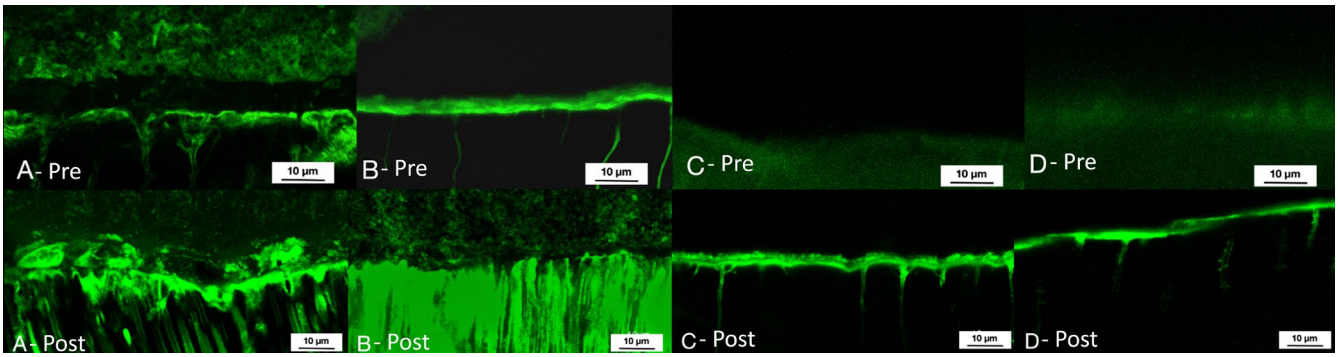

FIGURE 6 | Representative microscopy images showing fluorescence emitted from the different groups immediately after adhesion. (A-Pre) Group A preaging. (B-Pre) Group B preaging. (C-Pre) Group C preaging. (D-Pre) Extrafibrillar PAAN conditioned group D preaging. (A-Post) Group A post aging. (B-Post) Group B post aging. (C-Post) Group C post aging. (D-Post aging) Extrafibrillar PAAN conditioned group D post aging.

TABLE 1 | Mann–Whitney test and an ART (Aligned Rank Transformation) analysis showing effect between groups before and after aging, and cross-effects between groups for emitted fluorescence.

		Pre				Post			
p		**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Pre	A					0.0161**	0.0006***	0.0004***	<0.0001***
	B	0.1829				0.0001***	<0.0001***	0.0568	0.0004***
	C	<0.0001***	<0.0001***			<0.0001***	<0.0001***	<0.0001***	0.0003***
	D	<0.0001***	<0.0001***	0.1005		<0.0001***	<0.0001***	0.0007***	0.0916
Post	A								
	B					0.2385			
	C					<0.0001***	<0.0001***		
	D					<0.0001***	<0.0001***	0.1829	

Note: *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$.

the probability of adhesive failure increased with strength. The value of $\eta = 11.34$ MPa suggested that 63.2% of adhesive failures would occur at a strength of approximately 11.34 MPa. For the cohesive failures, the Weibull model could not be applied due to insufficient data (only 1 cohesive failure observation). For mixed failures, the value $\beta = 4.68$ indicates that the probability for this

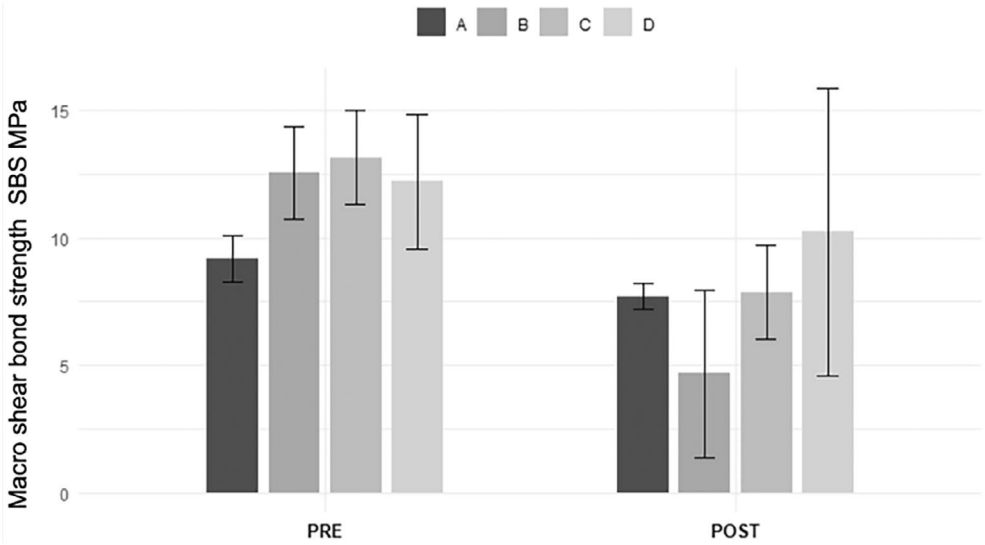

FIGURE 7 | Macro shear bond strength (SBS) values of each group, means, and standard deviation before and after aging.

type of failure is more concentrated and predictable compared to adhesive failures. Mixed failures become more likely at a later stage or at higher strengths. $\eta = 11.5$ MPa suggested that 63.2% of mixed failures occurred around this strength (Figures 8 and 9) (Table 4).

4 | Discussion

In the present study, the immediate adhesion forces between the groups did not show significant differences among them. Following the manufacturer's instructions in the treatment protocols, all groups exhibited similar behavior when subjected to shear forces after immediate adhesion. This finding was further substantiated by the SEM images, which revealed a uniformity in the interphase appearance initially formed among all groups, devoid of any gaps or voids. These findings do not support the proposed research hypothesis, as no significant differences were observed among the groups under immediate conditions. This finding aligns with the conclusions of several in vitro studies published in the literature, which evaluated immediate bond strengths to dentin [32, 33]. Subsequent to the aging of the samples, a statistically significant decrease was observed in the adhesion forces. The degradation of the hybrid layer over time is influenced by the substrate of adhesion, that is, the dentin, and the treatment applied to the dentin. As demonstrated in earlier studies, the degradation of adhesion forces and the loosening of the hybrid layer quality occur naturally due to the activity of matrix metalloproteinases (MMPs) and cysteine cathepsins (CTs), particularly with treatments involving phosphoric acid or self-etching systems [1, 2, 34, 35]. This observation was corroborated in the samples from our study. In contrast, the proteolytic activity was less pronounced in the groups treated with chelating agents such as EDTA combined with self-etch or PAANs. Moreover, in the group treated with polyacrylic acid salt, no significant differences were observed in adhesion forces and proteolytic activity before and after aging. This finding aligns with the conclusions of previous studies investigating extrafibrillar demineralization with chelating agents [17, 21, 25, 36, 37].

A detailed examination of the SEM images for groups A and B (Figure 3) revealed that aging induced marked changes in the morphology of the adhesive interface, compatible with hydrolytic degradation of the hybrid layer and enzymatic processes. Group B, in particular, displayed distinct gaps or voids along the interface. These observations are consistent with the proteolytic activity profile obtained in this study, which demonstrated unchanged enzymatic activity in group D following aging (Figures 3 and 4), moderate stability in group C (Figure 3), and increased proteolytic activity in groups A and B.

The results for adhesive and mixed failures suggest that the probability of failure increases with applied strength and becomes more predictable as strength increases. Research suggests that cohesive failures in dentin often account for a small percentage of the total number of failures. In most in vitro dentin bonding studies, the percentage of cohesive failures is very low compared to the adhesive or mixed, depending on the adhesive system evaluated and the protocol used [38]. However, there are cases where no cohesive failures are observed at all, that is, in studies with very efficient adhesive systems, when the intrinsic strength of the materials is high, or when the sample size is small. In the context of this study, the reason for the lack of cohesive failure could be a combination of the above factors. Figure 7 shows the probability of each type of failure modeled as a function of the strength achieved and, in agreement with previous studies [39]. It is observed that adhesive failure is more likely to occur at lower MPa strengths while mixed failure is more likely to occur at higher strengths.

The findings of this study demonstrate a marked decline in the post-aging adhesion strengths of groups that underwent conventional treatment with self-etch combined with 0.2% chlorhexidine, a synthetic protein inhibitor. Although studies have shown that 0.2% chlorhexidine inhibits enzyme activity after 10 years of aging in artificial saliva at 37°C [38], other studies suggest that a minimum of 2% should be used for this inhibition to be truly effective [39]. This could be one of the reasons why the results of group B exhibited the weakest post-aging performances in adhesion forces and zymography results. In studies employing

TABLE 2 | Data collected for the quantitative variables (by group pre/post aging): Mean, standard deviation, median, quartiles, minimum, and maximum.

	Mean				Std				Median				Quartiles Q1				Quartiles Q3				Minimum				Maximum			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Diameter	4.59	4.25	4.58	3.89	0.40	0.32	0.52	0.51	4.62	4.26	4.70	3.70	4.32	4.06	4.30	3.59	4.78	4.50	4.87	3.98	4.00	3.72	3.70	3.40	5.20	4.64	5.22	4.86
Ratio	2.29	2.11	2.29	1.94	0.20	0.17	0.26	0.26	2.31	2.10	2.35	1.85	2.16	2.01	2.15	1.79	2.39	2.23	2.44	1.99	2.00	1.86	1.85	1.70	2.60	2.32	2.61	2.43
Surface	16.64	14.04	16.61	12.04	2.89	2.20	3.63	3.34	16.76	13.85	17.34	10.75	14.65	12.72	14.54	10.09	17.97	15.60	18.63	12.48	12.56	10.86	10.75	9.07	21.23	16.90	21.39	18.54
N	74.10	60.87	82.83	63.62	17.48	28.44	16.57	26.63	75.00	71.50	80.90	70.00	66.15	43.00	72.25	52.23	77.45	78.13	94.15	72.27	50.00	13.50	58.00	16.70	106.50	96.96	107.60	109.00
Total Surface	8.61	7.02	8.31	6.02	1.28	1.10	1.81	1.67	8.67	6.92	8.67	5.37	7.78	6.36	7.27	5.04	9.26	7.80	9.31	6.24	6.92	5.43	5.37	4.54	10.61	8.45	10.69	9.27
Mpa	8.54	9.16	10.51	11.22	1.06	4.79	3.30	4.22	8.24	10.91	11.00	12.69	7.89	5.89	8.25	11.25	9.26	12.25	12.23	13.52	7.22	2.20	5.42	1.80	10.03	14.73	15.89	14.69

chlorhexidine as a synthetic inhibitor, it was observed that its application delayed the action of MMPs [32]. However, these studies utilized varying concentrations of chlorhexidine or different dentin treatments while maintaining the same concentrations of chlorhexidine. In contrast, the present study made a comparison between intrafibrillar or non-selective demineralization and extrafibrillar demineralization treatments.

Group A did not show statistically significant differences in bond strength before and after aging; however, differences in proteolytic activity were still observed. These findings are consistent with previous evidence suggesting that conventional non-selective demineralization strategies combined with protease inhibition may not fully prevent matrix metalloproteinase–mediated degradation over time. Nevertheless, adhesive systems combined with chlorhexidine continue to represent a widely accepted and clinically established approach for dentin bonding [11].

The aforementioned findings in the SBS strength values were aligned with the zymography results of this study, which revealed that there was no proteolytic activity in the extrafibrillar conditioned groups analyzed immediately after bonding, and the proteolytic activity detected after thermocycling was almost nonexistent along the hybrid layer compared to the groups analyzed with the conventional etch-and-rinse technique and the self-etch technique. These findings are consistent with those reported in previous studies [21, 34]. Conversely, augmented proteolytic activity was identified in the conventionally conditioned groups (etch-and-rinse and self-etch) immediately following bonding and subsequent thermocycling. The increase in activity after thermocycling was statistically significant, especially in the self-etch group, showing a 3-fold increase in the proteolytic activity ($p < 0.001$). The presence of proteolytic activity in conventionally treated groups has been documented in previous studies [34, 35, 40].

The prevailing literature posits that the protease activity of conventionally treated groups is activated by two factors: the low molecular size (smaller than 40 kDa, able to penetrate into the collagen fibers) and the low pH of the products used for conditioning. Proteases are activated and found in demineralized collagen poorly infiltrated by resin at the base of the hybrid layer [3–5, 21]. The intratubular activity observed in the zymograph analysis of certain samples in this study may be attributable to the precipitation of MMPs derived from dentinal fluid along the tubular wall during sample processing in the laboratory [21, 41].

Groups treated with chelating products with molecules of molecular weights greater than 40 kDa, such as the 220 kDa PAANs from this study, retain intrafibrillar minerals, providing an opportunity to eliminate the need for more technically sensitive wet bonding techniques. It is believed that the retention of these intrafibrillar minerals prevents endogenous fossilized proteases from being activated and contributing to the degradation of the collagen matrix [42]. These factors favor the long-term stability of the resin-dentin bond. The slightly alkaline pH of PAANs (pH 8.03–8.26) and EDTA (pH 8.0) may also help to preserve the activity of tissue inhibitors of MMPs and may have contributed to protecting the partially demineralized dentin collagen matrix from enzymatic degradation over time [21, 43].

TABLE 3 | ANOVA and Tukey HSD analysis showing effect between groups before and after aging, and cross-effects between groups for SBS forces.

		Pre				Post			
p		**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Pre	A					0.9683164	0.0853802	0.9743428	0.1960611
	B	0.2544783				0.0551229	0.0005336***	0.0407316*	0.9999642
	C	0.1136844	0.9997449			0.0225163*	0.0002063***	0.0151978*	1.0000000
	D	0.3642581	0.9999971	0.9963340		0.0855221	0.0008750***	0.0662407	0.9990470
Post	A								
	B					0.5356789			
	C					1.0000000	0.3936400		
	D					0.0443527*	0.0005706***	0.0340766*	

Note: *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$.

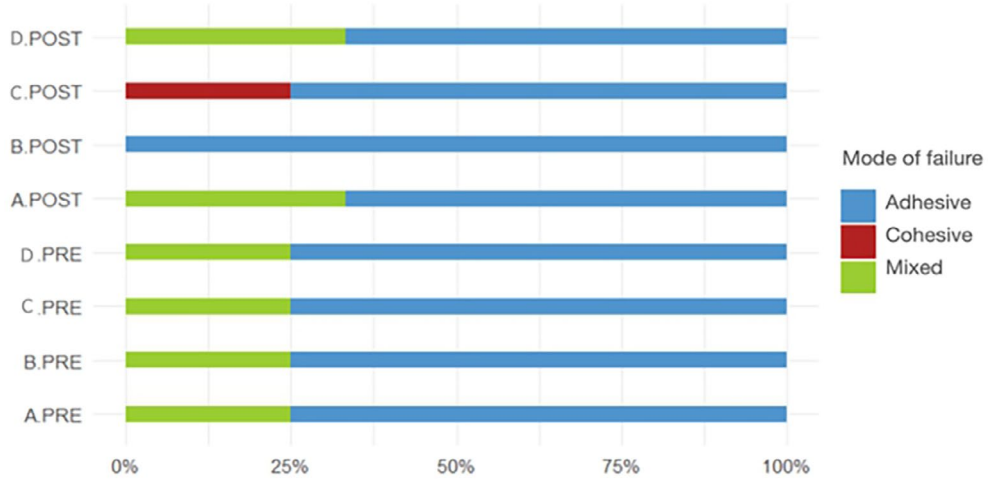

FIGURE 8 | Absolute and relative frequencies for qualitative variables.

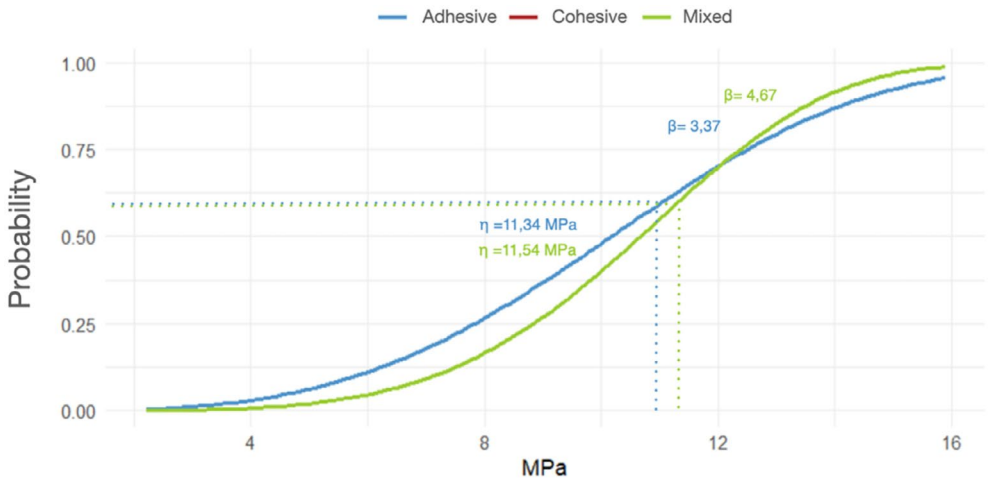

FIGURE 9 | Probability of each type of failure modeled depending on the achieved strength values.

Although promising results were obtained, there are limitations that prevent the authors from drawing definitive conclusions: the limited sample size, the heterogeneity among the treatment protocols, the intrinsic variability of dentin bond strength testing, and the in vitro nature of the study, which involve inevitable discrepancies between the simulated environments and the

TABLE 4 | Absolute and relative frequencies for qualitative variables.

Absolute frequency	A	B	D	E	Relative frequency	A	B	D	E
Adhesive	6	7	6	6	Adhesive	0.75	0.875	0.75	0.75
Cohesive	0	0	1	0	Cohesive	0	0	0.125	0
Mixed	2	1	1	2	Mixed	0.25	0.125	0.125	0.25

complex dynamics of the oral cavity. Within these limitations, the null hypothesis was accepted, since the groups treated with extrafibrillar demineralization strategies demonstrated greater preservation of adhesive interface stability and lower proteolytic activity after aging when compared with conventional dentin conditioning approaches.

Group A did not exhibit significant reductions in bond strength after aging despite showing increased proteolytic activity, which may suggest that enzymatic degradation of the hybrid layer can precede measurable mechanical deterioration. In contrast, the exclusive extrafibrillar demineralization strategy using PAANs demonstrated the greatest long-term stability, with minimal enzymatic activity and no significant reduction in bond strength after aging. These findings provide further insight into the potential role of extrafibrillar demineralization strategies in preserving adhesive bond strength and reducing enzymatic degradation over time, helping to clarify which dentin conditioning approaches may contribute to improved long-term stability of the resin–dentin interface. Further research is needed to fill the knowledge gap more precisely.

5 | Conclusions

Regarding the first research hypothesis, the initial bond strength of the adhesive systems to dentin did not differ between extrafibrillar and non-selective demineralization methods. After accelerated aging, significant reductions in bond strength were observed, particularly in the self-etch adhesive group. In contrast, the PAANs-treated group showed no statistically significant reduction after aging, indicating greater long-term bond stability compared with conventional conditioning protocols combined with protease inhibition.

Concerning the second research hypothesis, higher gelatinolytic activity was detected in the groups treated with conventional etching protocols before and after accelerated aging. In contrast, extrafibrillar demineralization treatments with EDTA and especially with PAANs showed reduced enzyme activity both immediately and after aging.

These findings suggest a potential advantage of chelate-based conditioning approaches for maintaining adhesive interface stability; however, the contribution of conditioning strategy, adhesive systems, and protease inhibitors should be considered when interpreting the results.

Author Contributions

Ana Torres Muñoz: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, visualization. **José F. Bartolomé:** conceptualization, data curation, resources, writing – review and editing, funding acquisition, visualization, supervision. **Carlos Oteo Calatayud:** methodology, investigation, supervision, writing – review and editing. **Guillermo J. Pradíes Ramiro:** validation, data curation, writing – review and editing, methodology, resources, investigation. **Óscar González-Martín:** conceptualization, validation, data curation, resources, investigation, methodology, writing – review and editing.

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Disclosure

The authors have nothing to report.

Ethics Statement

The present study constitutes an in vitro experimental investigation, which was approved by the Research Ethics Committee of the Hospital Clínico San Carlos under number 22/696-E_Tesis.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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