Received: 14 September 2021 | Revised: 12 October 2021 | Accepted: 1 November 2021

DOI: 10.1111/odi.14067

ORAL DISEASES Leading in Oral, Maxillofacial, Head & Neck Medicine WILEY

ORIGINAL ARTICLE

Virucidal activity in vitro of mouthwashes against a feline coronavirus type II

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Abstract

Transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can occur through saliva and aerosol droplets deriving from the upper aerodigestive tract during coughing, sneezing, talking, and even during oral inspection or dental procedures. The aim of this study was to assess in vitro virucidal activity of commercial and experimental mouthwashes against a feline coronavirus (FCoV) strain. Commercial and experimental (commercial-based products with addition of either sodium dodecyl sulfate (SDS) or *thymus vulgaris* essential oil (TEO) at different concentrations) mouthwashes were placed in contact with FCoV for different time intervals, that is, 30 s (T30), 60 s (T60), and 180 s (T180); subsequently, the virus was titrated on Crandell Reese Feline Kidney cells. An SDS-based commercial mouthwash reduced the viral load by 5 log10 tissue culture infectious dose $(TCID)_{50}$/50 μl at T30 while a cetylpyridinium (CPC)-based commercial mouthwash was able to reduce the viral titer of 4.75 log10 at T60. Furthermore, five experimental mouthwashes supplemented with SDS reduced the viral titer by 4.75–5 log10 according to a dose- (up to 4 mM) and time-dependent fashion.

KEYWORDS
dental practice, feline coronavirus, in vitro, mouthwashes, virucidal activity

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for coronavirus disease 2019 (COVID-19), has rapidly spread across the globe generating profound effects in the human health and behavior and in global economy.

Coronaviruses are a group of enveloped RNA viruses with a typical "crowned" structure with the "spike protein" in its membrane envelope (Zhu et al., 2020). The target of the SARS-CoV-2 spike protein is the angiotensin-converting enzyme 2 (ACE2) receptor, which is responsible for the entry of the virus into cells (Chen et al., 2020). ACE2 receptor is widely expressed in different tissues and organs, that is, mucosal tissues, gingiva, non-keratinizing squamous epithelium, and tongue and salivary gland epithelial cells (Hamming et al., 2004). As SARS-CoV-2 has been detected in saliva samples (To et al., 2020), viral transmission can potentially occur via interactions with saliva and aerosol droplets deriving from oropharynx during coughing, sneezing, talking, and even during oral inspection or dental procedures (Li et al., 2020).

Facial masks, facial barriers, and gloves can protect from the viral transmission, but dental procedures can produce a high generation

of aerosols overcoming physical barriers. High-speed dental handpieces and ultrasonic tips, typically used for common dental procedures like caries treatment or tartar removal, produce aerosols that can also remain suspended in the air for long periods and contaminate surfaces with a potential subsequent transmission risk. It is possible to substitute this protective equipment with manual instruments and/or anti-retraction handpieces and, when possible, use rubber dam, but potential risk remains (Gandolfi et al., 2020; Prati et al., 2020).

Besides physical barriers, another approach is the adoption of chemical-based detergents to inactivate SARS-CoV-2 on surfaces and biological tissues (Peng et al., 2020). Handwash is a well-documented procedure that is able to reduce the risk of SARS-CoV-2 spread by contact transmission (Jing et al., 2020).

Mouthwashes are widely used products for personal care due to their ability to reduce the number of microorganisms in the oral cavity (Koletsi et al., 2020). Originally used as adjuvants to control microbial growth in the oral cavity (Jenkins et al., 1994), preoperative antiseptic mouth rinses have also been recommended to lower down microbial load in the aerosols and drops released during oral surgery procedures (Peng et al., 2020). However, to date, there is still no clinical evidence that they can prevent SARS-CoV-2 transmission.

Peng et al. recommended preprocedural mouth rinse containing oxidative agents such as 1% hydrogen peroxide (H_2O_2) or 0.2% povidone–iodine (PVP-I) (Peng et al., 2020). PVP-I 0.5% completely inactivate SARS-CoV-2 after 15-s contact (Bidra et al., 2020a). American Dental Association (2020), Centers for Disease Control and Prevention (2020), and national guidelines recommend mouth rinses for 30 s by using the following: (i) 1.5% or 3% H_2O_2 15 ml; (ii) 0.2% or 0.4% or 0.5% PVP-I 9 ml; (iii) 0.12% chlorhexidine (CHX) 15 ml; and (iv) 0.05% cetylpyridinium (CPC) 15 ml. More recent guidelines recommend a first mouth rinse for 30 s with 1% H_2O_2 or 0.2% PVP-I or 0.05% CPC and a second mouth rinse with 0.2–0.3% CHX for 60 s (Kariwa et al., 2006; Meng et al., 2020). CPC-based mouthwashes significantly reduced SARS-CoV-2 infectivity being able to disrupt the integrity of viral envelope (Muñoz-Basagoiti et al., 2021). Furthermore, a decreased salivary viral load was reported in SARS-CoV-2–positive patients using CPC and PVP-I–based mouthwashes (Seneviratne et al., 2021).

The aim of this study was to evaluate the virucidal activity in vitro of commercial and experimental mouthwashes against a feline coronavirus (FCoV) type II strain.

FCoV-II belongs to the Alphacoronavirus genus, while SARS-CoV-2 is a member of the Betacoronavirus genus. Both viruses, despite having a limited genetic correlation (Sharun et al., 2020), exhibit overlapping physicochemical features belonging to the same viral family. This study is focused on the evaluation of the sensitivity/resistance features of the virus to virucidal substances, thus effecting the structural components. Accordingly, the use of FCoV was considered suitable. Other reasons for the choice of FCoV are herein reported:

(i) lack of any biological risk for operators as FCoV is not pathogenic for humans;
(ii) ability of inducing rapid and evident in vitro cytopathic effect (cpe), thus allowing an easy and rapid quantification of viral titer and, consequently, the live virus unlike the use of molecular assays (PCR and real-time PCR), which evaluate viral genome copies.

2 | MATERIALS AND METHODS

2.1 | Mouthwashes (M)

The following commercial mouthwashes were used:

M1: (main component: CHX digluconate 0.2%);
M2: (main component: ethanol + essential oils (EOs) eucalyptol 0.091% w/v, thymol 0.063% w/v, and menthol 0.042% w/v);
M3: (main component: sodium dodecyl sulfate (SDS) + EOs eucalyptol 0.091% w/v, thymol 0.063% w/v, and menthol 0.042% w/v);
M4: (main component: CPC chloride 0.1%);

Commercial mouthwashes that did not show significant virucidal activity were supplemented with different concentrations of *thymus vulgaris* essential oil (TEO) (Specchiasol, Bussolengo, Verona, Italy) (Catella et al., 2021) or SDS (Sigma-Aldrich) (data not shown) based on expected antiviral activity. Therefore, the following experimental mouthwashes were also tested:

M5: (M1 + TEO 3000 μg/ml);
M6: (M1 + TEO 30,000 μg/ml);
M7: (M2 + 4 mM SDS);
M8: (M2 + 3 mM SDS);
M9: (M2 + 2 mM SDS);
M10: (M2 + 1 mM SDS);
M11: (M2 + 0.5 mM SDS).

2.2 | Viruses and cell cultures

The virucidal activity was evaluated against the FCoV type II (FCoV II) strain 25/92 (Buonavoglia et al., 1995).

FCoV type II strain was used as surrogate of SARS-CoV-2 for several reasons: (i) lack of any biological risk for operators as FCoV is not pathogenic for humans; (ii) FCoV belongs to the *Coronaviridae* family (such as SARS-CoV-2); and (iii) FCoV-II induces rapid and evident cytopathic effect (cpe) in vitro.

The virus was cultured on Crandell Reese Feline Kidney (CRFK), using Dulbecco's minimal essential medium (D-MEM) with 10% fetal bovine serum.

The viral titer of stock virus used for virucidal activity assays was 5.50 tissue culture infectious doses ($TCID_{50}$/50 μl).

2.3 | Virucidal activity assay

The viral stock (1 ml) was placed in contact at room temperature with the same amount of each mouthwash. After 60-s (T60) and

180-s (T180) contacts at room temperature, samples were 10-fold diluted from 10^{-1} to 10^{-6} using DMEM. The undiluted mixture and each dilution were dispensed into 96-well microtiter plates using 4 wells for each dilution (100 µl/well).

CRFK cells suspended in D-MEM with 10% fetal bovine serum (20,000 cells/100 µl/well) were then added to each well.

Control virus, used for the tests and maintained for 60 and 180 s under the same conditions as mouthwashes/virus, was also titrated.

The plates were incubated for 72 h at 37°C in an incubator with 5% CO_2. On the basis of the cpe, the titer was calculated using the Karber formula (Kärber, 1931).

Mouthwashes M3, M4, M7, M8, and M9, resulting in a reduction of viral titer of 4.75 log 10 at T60 and T180, were chosen for the evaluation of virucidal activity also at 30-s (T30) contact.

Bovine serum albumin (BSA), as an interfering substance, was added to the mouthwashes/control solutions to mimic environmental contamination.

All the experiments were performed in triplicate.

2.4 | Data analysis

Normality of distribution was assessed by Shapiro–Wilk test. Data from virucidal activity of mouthwashes were assessed by Student's *t*-test for independent samples (statistical significance set at 0.05). Statistical analyses were performed with the software GraphPad Prism v 8.0.0 (GraphPad Software).

3 | RESULTS

In the virucidal activity assays, control virus did not show significant variations in the viral titers at different time intervals as evaluated on CRFK cells.

Preliminary experiments performed with mouthwashes (without FCoV) on CRFK cells evidenced cytotoxic effect in undiluted solutions. Cytotoxic effects were often observed in the wells containing undiluted mixture composed of mouthwashes and virus and in some cases M4, also in the wells containing the 10^{-1} dilution of the mouthwash/virus mixture.

The results of viral titrations on CRFK cells at T30, T60, and T180 contacts of the mouthwashes with the FCoV were reported in Figure 1. Moreover, virucidal activity of the mouthwashes against FCoV was statistically compared with virus control and reported in Table 1.

M1 and M2 did not reduce or slightly reduce (0.25 log 10) viral titers, respectively, as compared to those of the control virus at T60 and T180 (5 log 10).

Despite the presence of cytotoxic effects in the wells inoculated with the undiluted mixture and partially in those inoculated with the 10^{-1} dilution that hampered the ability to verify the possible presence of cpe, M3 significantly reduced the viral titer of 5.00 log10 at T30 ($p < 0.0001$) and 4.75 log 10 at T60 and T180 ($p < 0.0001$) while M4 significantly reduced viral titers of 3.00 log 10 at T30 ($p = 0.0001$) and 4.75 log 10 at T60 and T180 ($p < 0.0001$). Both results were compared to the respective virus controls at T30 (5.50 log 10) and T60 and T180 (5.25 log 10).

M5 did not show any significant reduction in viral titer at T60 and T180 as the titer of the mouthwash/virus mixture was identical to that of the control virus at the respective time intervals.

M6 consistently reduced the viral titers of 2.50 log 10 at T60 and T180 ($p = 0.0003$) as compared to the control virus (5 log 10) at the respective time intervals.

Despite the presence of cytotoxic effects in the wells inoculated with the undiluted mixture, M7 and M8 showed a consistent reduction in viral titer of 5 log 10 at T30 ($p < 0.0001$) and 4.75 log 10 T60 and T180 ($p < 0.0001$) while M9 reduced viral titer of 3.75 log 10 at T30 ($p < 0.0001$) and 4.75 log 10 at T60 and T180 ($p < 0.0001$) as compared to the respective time interval of the virus control.

M10 determined a significant reduction in viral titer of 4.00 log 10 at T60 ($p < 0.0001$) and 4.75 log 10 and T180 ($p < 0.0001$), while M11 reduced the viral titer of 1.50 at T60 ($p = 0.0018$) and 4.75 log 10 at T180 ($p < 0.0001$), with respect to virus control at the different time intervals.

The experiments performed using BSA did not exhibit significant differences as compared to experiments without BSA.

4 | DISCUSSION

Various compounds were used in mouthwashes for treatment and/or prevention of oral bacterial infection, such as periodontal diseases and postsurgical infections (Jenkins et al., 1994). The interaction and efficacy of mouthwashes on viruses were historically neglected, but actually mouthwashes have been addressed as a potential tool for the prevention of SARS-CoV-2 (Carrouel, Gonçalves, et al., 2021).

Preprocedural mouthwashes containing oxidative agents such as H_2O_2 or PVP-I have been suggested to reduce the salivary viral load of SARS-CoV-2 (Elzein et al., 2021; Peng et al., 2020).

H_2O_2 liberates oxygen-free radicals and disrupts viral lipid envelope (O'Donnell et al., 2020; Peng et al., 2020), and as reported in in vitro studies, coronaviruses and influenza viruses were most sensitive to 3% H_2O_2 within 1–30 min (Mentel' et al., 1977). Moreover, H_2O_2-based mouthwashes are safe for mucous membranes, even when H_2O_2 is used at a concentration of 3% over 6 min (Caruso et al., 2020). Moreover, 1.5% H_2O_2-based mouthwash significantly reduced SARS-CoV-2 viral load up to 30 min after rinsing (Eduardo et al., 2021).

PVP-I disrupt proteins and oxidize nucleic acid structures with free iodine dissociation, and it has higher virucidal activity against both enveloped and nonenveloped viruses (Pattanshetty et al., 2021).

In a recent study, a comparison of in vitro inactivation of SARS-CoV-2 with H_2O_2 and PVP-I was reported. After 15 and 30 s of contact time, PVP-I completely inactivated the virus at the concentrations of 0.5%, 1.25%, or 1.5% (Bidra et al., 2020a). Conversely, H_2O_2 (1.5% and 3%) showed minimal virucidal activity in the same contact time (Bidra et al., 2020b). A comparison of PVP-I (0.5%, 1%, and 1.5%) and 70% ethanol was reported showing that after 15 s of contact, PVP-I completely inactivated

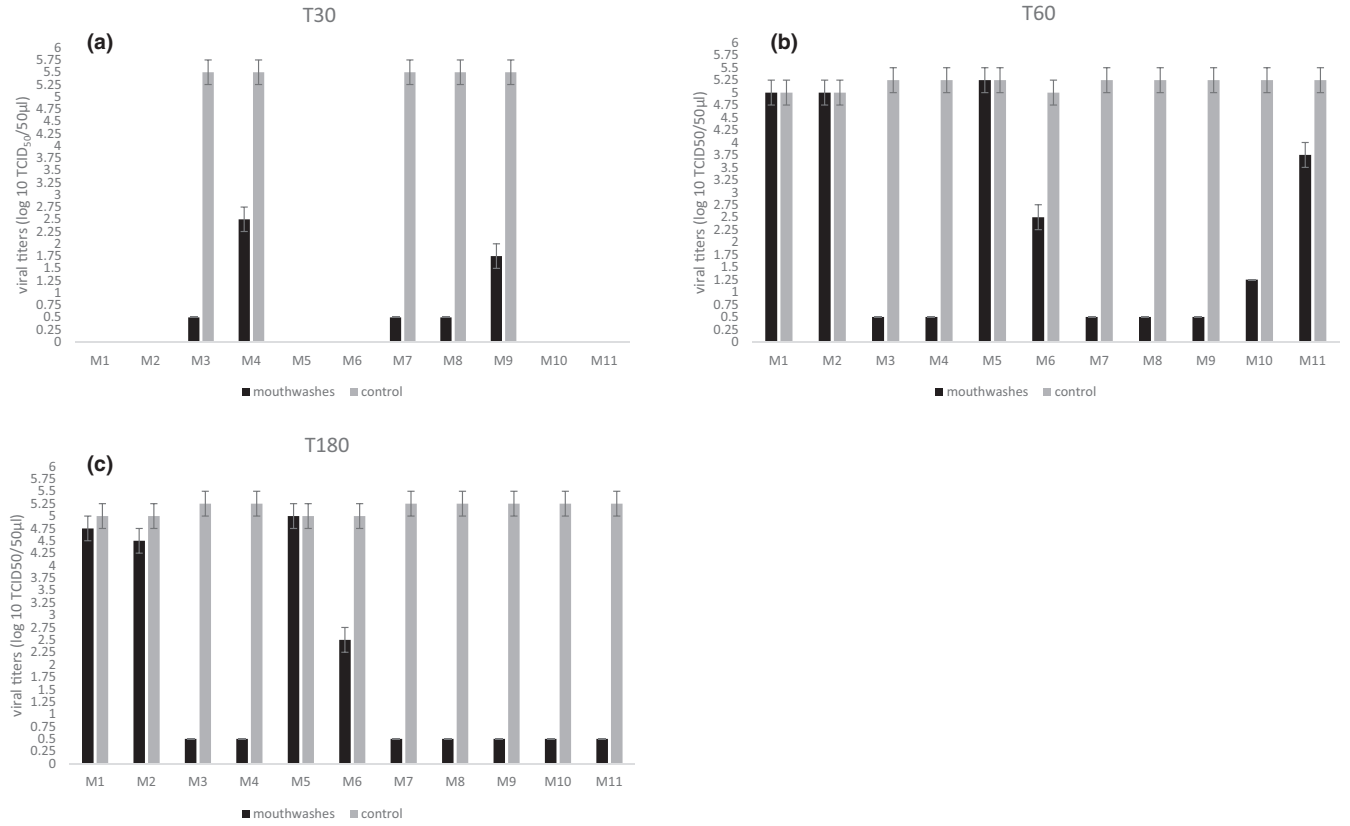

FIGURE 1 In vitro evaluation of the virucidal activity of mouthwashes against feline coronavirus type II after contact time of 30 s (T30) (a), 60 s (T60) (b), and 180 s (T180) (c). M1: (main component: CHX digluconate 0.2%); M2: (main component: ethanol + essential oils (EOs) eucalyptol 0.091% w/v, thymol 0.063% w/v, and menthol 0.042% w/v); M3: (main component: sodium dodecyl sulfate (SDS) + EOs eucalyptol 0.091% w/v, thymol 0.063% w/v, and menthol 0.042% w/v); M4: (main component: cetylpyridinium 0.1%); M5: (M1 + TEO 3000 µg/ml); M6: (M1 + TEO 30,000 µg/ml); M7: (M2 + 4 mM SDS); M8: (M2 + 3 mM SDS); M9: (M2 + 2 mM SDS); M10: (M2 + 1 mM SDS); and M11: (M2 + 0.5 mM SDS)

SARS-CoV-2 whereas ethanol 70% inactivated the virus after 30 s of contact (Bidra et al., 2020a).

Using real-time reverse transcriptase PCR (RT-qPCR) to analyze the effect of gargling in the mouth and throat with 20 ml of H_2O_2 1% for 30 s, no significant decrease in SARS-CoV-2 viral load was observed (Gottsauner et al., 2020).

PVP-I appear more effective for viral inactivation. PVP-I at 0.5% for 15 s reduce SARS-CoV-2 load by 4 log 10, whereas application for 30 or 60 s reduces the load by more than 5 log 10 (Hassandarvish et al., 2020).

Moreover, the use of 15 ml of 1% PVP-I mouthwash for 1 min significantly reduced the SARS-CoV-2 titer in the saliva for 3 h, as evaluated by RT-qPCR (Martínez Lamas et al., 2020).

However, the use of PVP-I is contraindicated in patients with allergy to iodine, with thyroid disease, pregnancy, or treatment with radioactive iodine (Gray et al., 2013).

In this study, the virucidal effects of several mouthwashes were evaluated in vitro against FCoV at different contact times, ranging from 30 s to 1 min and to 3 min, which are the common times for application of mouthwashes (Jenkins et al., 1994). A commercial CHX-based mouthwash (M1), tested in this study, appeared ineffective against FCoV. Virucidal effects at 0.2% concentration were not observed at T60 and T180. CHX, a cationic bisbiguanide, induces bacterial and fungal lysis increasing the permeability of the cell wall (da Costa et al., 2017). In vitro effects have been reported against enveloped viruses (Bernstein et al., 1990; Elzein et al., 2021). In this study, a commercial CPC-based mouthwash (M4) used at a concentration of 0.1% was able to reduce viral titer by 3 log 10 at T30 and 4.75 log 10 at T60 and T180. CPC, a cationic quaternary ammonium compound, is used as an alternative to CHX (Feres et al., 2010). The antiviral effect of CPC against coronaviruses is probably based on its lysosomotropic activity and its ability to destroy viral capsids (Baker et al., 2020). Eduardo et al. (2021) described the efficacy of CPC and CHX-based mouthwashes in reducing SARS-CoV-2 viral load in saliva up to 60 min after rising.

A commercial ethanol and EO-based mouthwash (M2) was also tested in this study. Ethanol is an excipient used in various mouthwashes, and it is active at high concentration on the inactivation of enveloped viruses, by dissolving the lipid membrane and denaturing the proteins (Jing et al., 2020). In this study, M2 did not show virucidal effects at T60 and T180. In a previous report, ethanol at a 70% concentration was able to inactivate SARS-CoV-2 after a 30-s contact (Bidra et al., 2020b). Many proprietary mouthwashes contain alcohol (ethanol), and in some products the concentration of ethanol

TABLE 1 Virucidal activity of mouthwashes (M1 to M11) against feline coronavirus evaluated in CRFK cells after a contact time of 30 s (T30), 60 s (T60), and 180 s (T180) compared with control virus (CV)

	Viral titers (log10 $TCID_{50}$/50 μl)								
	T30			T60			T180		
Comparisons	MDV	95% CI	*p* value	MDV	95% CI	*p* value	MDV	95% CI	*p* value
CV versus M1	NA	NA	NA	0	[−0.567; 0.567]	>0.9999 ns	0.25	[−0.817; 0.317]	0.2879 ns
CV versus M2	NA	NA	NA	0	[−0.567; 0.567]	>0.9999 ns	0.50	[−1.067; 0.067]	0.0705 ns
CV versus M3	5.00	[−5.567; −4.433]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M4	3.00	[−3.567; −2.433]	0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M5	NA	NA	NA	0	[−0.567; 0.567]	>0.9999 ns	0	[−0.567; 0.567]	>0.9999 ns
CV versus M6	NA	NA	NA	2.50	[−3.067; −1.933]	0.0003[b]	2.50	[−3.067; −1.933]	0.0003[b]
CV versus M7	5.00	[−5.567; −4.433]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M8	5.00	[−5.567; −4.433]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M9	3.75	[−4.317; −3.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M10	NA	NA	NA	4.00	[−4.567; −3.433]	<0.0001[b]	4.75	[−5.317; −4.183]	<0.0001[b]
CV versus M11	NA	NA	NA	1.50	[−2.067; −0.933]	0.0018[a]	4.75	[−5.317; −4.183]	<0.0001[b]

Abbreviations: 95% CI, 95% confidence interval; MDV, mean difference of viral titers; NA, not assessed; ns, not significant.

[a]Very significant.

[b]Highly significant.

can be as high as 26% (Lachenmeier, 2008). Accordingly, in this report we could hypothesize the presence of ethanol at low concentration, unable to inactivate the virus. M2 also contained different EOs in the mixture. EOs, volatile and odorous products extracted from the plants, have antiseptic properties. EOs have been demonstrated to interfere with the viral envelope, thus provoking its dislocation (Wińska et al., 2019). Antiviral effects of EOs in in silico studies have been reported by (Silva et al., 2020). Furthermore, mouthwashes containing EOs demonstrated virucidal activity on SARS-CoV-2 in vitro (Xu et al., 2021). Other in vivo and in vitro studies reported the efficacy of cyclodextrin- and citrox- or anionic phthalocyanine derivative-based mouthwashes against SARS-CoV-2 (Carrouel, Valette, et al., 2021; Santos et al., 2021).

To evaluate the virucidal effect of EOs, two experimental mouthwashes (M5 and M6) were prepared mixing M1 (0.2% CHX) with TEO at two different concentrations. In a previous report, TEO demonstrated in vitro antiviral and virucidal effects against FCoV (Catella et al., 2021), used as a surrogate model for the study of antivirals against CoVs. M5 containing 3000 μg/ml of TEO did not exhibit virucidal effects at any time interval, while M6 containing TEO at a concentration of 30,000 μg/ml reduced the viral titer by 2.50 log 10 at T60 and T180.

In this study, the virucidal effect of a proprietary SDS and EO-based mouthwash (M3) was also assessed against FCoV. SDS, an anionic surfactant, is generally included in toothpastes, and it is known for its efficiency to dissolve the outer layer of viruses and bacteria, while the hydrophilic side dissolves in water, acting as an emulsifier (Jahromi et al., 2020). Anionic surfactants contained in hand soap disrupt the envelope layer of SARS-CoV-2 after long enough contact time (Jahromi et al., 2020). SDS interacts with cell membranes, elevating the intracellular Ca^{2+} influx with an increase in intracellular reactive oxygen species (ROS) and IL-1α production. ROS increase could also interfere with viral replication, with an indirect cellular stimulation (Mizutani et al., 2016).

M3 reduced viral titer by 4.75–5 log10 consistently at any time intervals. Also, in this report, we assessed the virucidal effects of experimental M2-based mouthwashes, namely, M7, M8, M9, M10, and M11, containing decreasing concentrations of SDS (4, 3, 2, 1, and 0.5 mM, respectively). M7 and M8 consistently decreased the viral titer by 4.75–5 log 10 at any time intervals, while M9, M10, and M11 reduced the viral titers with a dose- and time-dependent fashion.

5 | CONCLUSIONS

In our study, the most promising mouthwashes were those based on SDS. Both commercial (M3) or experimental (M7 to M11) SDS-based mouthwashes showed virucidal effects at all the tested contact times. Interestingly, a decrease in SDS concentrations in the mouthwashes affected the ability to inactivate the virus. The SDS concentration was not reported originally in the commercial SDS-based mouthwash label; it is likely that the SDS concentration was over 2 mM, considering the virucidal effects of the SDS-based M9, M10, and M11.

Another commercial mouthwash, M4, showed promising results. M4 contained CPC 0.1% and required a longer contact time than SDS-based commercial or experimental mouthwashes tested in our study.

Commercial (M1 and M2) and experimental (M5 and M6) CHX 0.2% and ethanol + EO-based mouthwashes appeared ineffective against FCoV. Adding TEO at 30,000 μg/ml to CHX 0.2% revealed promising virucidal properties, even if with lower efficacy than other

mouthwashes. Accordingly, CHX should be used in combination with other virucidal compounds as suggested by recent guidelines (Meng et al., 2020).

The results reported in this study are speculative and preliminary as obtained using a feline coronavirus strain. However, since coronaviruses share similar physicochemical features, further in vitro and in vivo studies using SARS-CoV-2 are encouraged to confirm the virucidal effects of SDS-, CPC-, and TEO-based mouthwashes in the oral cavity.

Another pivotal aim will be to determine the duration of a rinse required to reduce the viral titer. This would allow the estimation of the right time for repeating a rinse to avoid the risk of SARS-CoV-2 transmission.

ACKNOWLEDGMENTS

Open Access Funding provided by Universita degli Studi di Bari Aldo Moro within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Alessio Buonavoglia: Conceptualization; Writing-original draft. **Michele Camero:** Methodology. **Gianvito Lanave:** Formal analysis; Software. **Cristiana Catella:** Methodology. **Claudia Maria Trombetta:** Visualization. **Maria Giovanna Gandolfi:** Validation. **Gerardo Palazzo:** Methodology. **Vito Martella:** Resources; Supervision. **Carlo Prati:** Supervision; Writing-review & editing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.14067.

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How to cite this article: Buonavoglia, A., Camero, M., Lanave, G., Catella, C., Trombetta, C. M., Gandolfi, M. G., Palazzo, G., Martella, V., & Prati, C. (2021). Virucidal activity in vitro of mouthwashes against a feline coronavirus type II. *Oral Diseases*, 00, 1–8. https://doi.org/10.1111/odi.14067