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Antibacterial and Antifungal Capacity of Three Commercially Available Mouthwashes with Different Concentrations of Chlorhexidine

Efecto antibacteriano y antifúngico de tres enjuagues bucales comerciales con diferentes concentraciones de clorhexidina

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ABSTRACT: Chlorhexidine was introduced almost seven decades ago and has a myriad of applications in dentistry. Few studies have evaluated the antimicrobial and antifungal capacity of different concentrations of chlorhexidine mouthwashes. Therefore, the aim of this study, was to evaluate *in vitro*, the antibacterial and antifungal capacity of three commercially available mouthwashes in Costa Rica, with different concentrations of chlorhexidine, 0.12%, 0.06%, and 0.03%. The experimental method selected was the Kirby-Bauer method to evaluate the antibacterial and antifungal effect of each compound by measuring the inhibitory effect on *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Candida albicans strains*, exposed to the antiseptic solutions. All samples showed some degree of antibacterial and antifungal effect. Even though we provide *in vitro* results, our findings are of relevance since all the species used in our experiment are microorganisms that may be present in dental plaque. Our results further support evidence that oral hygiene regimens may include mouthwashes with low doses of chlorhexidine and maintain reasonable antibacterial and antifungal efficacy.

KEYWORDS: Chlorhexidine; Mouthwashes; Antibacterial effect; Low-dose concentration mouthwash.

RESUMEN: La clorhexidina se introdujo hace casi siete décadas y tiene una gran variedad de aplicaciones en odontología. Pocos estudios han evaluado la capacidad antimicrobiana y antifúngica de diferentes concentraciones de enjuagues bucales con clorhexidina. Por lo tanto, el objetivo de este estudio fue evaluar *in vitro*, la capacidad antibacteriana y antifúngica de tres enjuagues bucales disponibles comercialmente en Costa Rica, con diferentes concentraciones de clorhexidina, 0.12%, 0.06% y 0.03%. El método experimental seleccionado fue el método Kirby-Bauer para evaluar el efecto antibacteriano y antifúngico de cada compuesto midiendo el efecto inhibidor sobre *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* y *Candida albicans*, expuestos a la solución antiséptica. Todas las muestras mostraron algún grado de efecto antibacteriano y antifúngico. Aunque proporcionamos resultados *in vitro*, nuestros hallazgos son de relevancia, ya que todas las especies utilizadas en nuestro experimento son microorganismos que pueden estar presentes en la placa dental. Nuestros resultados respaldan aún más la evidencia de que los regímenes de higiene bucal pueden incluir enjuagues bucales con dosis bajas de clorhexidina y mantener una eficacia antibacteriana y antifúngica razonable.

PALABRAS CLAVE: Chlorhexidina; Enjuagues bucales; Efecto antibacteriano; Enjuague bucal de baja concentración.

INTRODUCTION

Chlorhexidine (CHX) is the most commonly used broad-spectrum antiseptic agent in oral hygiene. It is widely prescribed in different dental fields, especially in patients that cannot perform correctly mechanical biofilm control, due to physical/mental impairment, lack of motivation or xerostomia (1). Additionally, CHX has multiple applications in dentistry because of its substantivity. CHX is adsorbed by the oral mucosa, oral proteins, and onto hydroxyapatite of the dental surface (2). This adsorbed CHX is gradually released at effective doses that guarantee the persistence of its antibacterial activity. CHX is available commercially as mouthwash, gel and aerosol spray. For professional use slow-release disks or chips of CHX are available (1).

Mouthwashes containing CHX have been reported to be effective in oral hygiene regimens, as adjuvant in the management of gingivitis, periodontitis and peri-implant disease. Dental plaque is the etiological factor of gingivitis and periodontitis. Mouthwashes are an ideal vehicle to incorporate agents such as CHX that has demonstrated to have a positive effect preventing plaque accumulation and gingival inflammation (3). Commercially, these therapeutic agents are available in wide range of concentrations, or combined with secondary agents to enhance its clinical effect. In general, chlorhexidine mouthwashes can be classified as bactericidal or therapeutic when concentrations above 0,12% are used, or low-dose concentration mouthwashes for more diluted presentations (1). Figure 1 summarize some of the particular characteristics and indications (Figure 1.)

Low dose vs therapeutic Chlorhexidine mouthwashes

	Low dose chlorhexidine mouthwash	Therapeutic chlorhexidine mouthwash
Effect	Bacteriostatic	Bactericidal effect
CHX Concentrations	0,03% - 0,06%	0,12% - 0,2%
Side effects	Lower	Increased (especially after prolonged use)
Antiseptic efficacy	Mild	High
Indications	Prolonged treatment (+ 2 weeks)	Acute therapeutic interventions (10-14 days)
Clinical Settings	Orthodontic treatment coadjutant, disabled patient, use of intraoral orthopedic appliances, chronic halitosis, use of removable/fixed prosthetics, maintenance periodontic treatment, presence of chronic intraoral pathologies, increased caries risk (i.e. radiotherapy, cariogenic diet, motor disability).	Post-surgical antiseptic support, gingivitis/periodontitis treatment, oral/dental acute trauma, acute oral bacteremia, preoperative antiseptic interventions (i.e. dental aerosol disinfections)

Figure 1. General classification, characteristics and clinical indications of Chlorhexidine mouthwashes.

Robust scientific literature has established that patients with gingivitis, have significant reductions in plaque and gingivitis scores with mechanical oral hygiene and CHX mouthwash (4). Additionally, CHX can break up existing plaque, inhibit plaque regrowth and reduce gingival inflammation (4,5). In patients with inadequate self-performed plaque control during supportive periodontal therapy, a low CHX concentration mouth rinse (0.05%) combined with 0.05% cetyl-pyridinium chloride (an antiplaque agent) showed significant reductions of microbial loads in saliva and in the gingival sulcus (6). Specifically subgingival counts of *Fusobacterium nucleatum* and *Prevotella intermedia* decreased when compared to placebo (6). Adding fluoride to CHX mouthwash (0.1% sodium fluoride and 0.2% CHX) significantly decreased salivary *S. mutans* count after two weeks in 12-14 year-old students (7). The combination of these two chemotherapeutic are beneficial for certain high-risk groups for the prevention of caries and gingivitis. A meta-analysis evaluated the effect of chlorhexidine mouthwash as an adjunct to mechanical therapy in periodontal patients and found a slight reduction in probing depths and a slight effect on clinical attachment gain (9).

CHX may also be used in the treatment of halitosis by reducing halitosis-related bacteria. Mouthwashes containing low doses of CHX (0.05%), 0.05% cetylpiridinium chloride and 0.14% zinc lactate have shown to reduce organoleptic scores and volatile sulphur compounds (VSC) in patients with oral malodor (10,11). The use of CHX (0.02%) mouth rinse alone, also produced a significant reduction in VSC and organoleptic scores (11,12). Similar results with CHX at a concentration of 0.12% were reported in combination with mechanical brushing (13,14). A mouth wash that combined zinc at 0.3%, and chlorhexidine at a low concentration of 0.025%, was efficient in removing VSC, suggesting a synergistic mode of action of these two components (15). In the treatment of halitosis, adjuvants, such as chlorhexidine mouthwashes do not substitute the medical management of the cause.

CHX has been recommended preoperatively and postoperative after oral surgery to avoid and associated complications. Before oral surgery, CHX is indicated for surgical hand antisepsis to reduce infection since there is evidence to reduce bacterial load on skin of the clinician and patient's surgical site (18). As stated before, CHX controls biofilm, therefore it is used during the peri-operative period, before and after oral surgery procedures (1). CHX is suggested during healing phase, in cases where regular biofilm control procedures

cannot be performed, since these would interfere with wound healing. The convenience of using CHX after oral surgery is due to its capacity of preventing recolonization of bacteria at the surgical site and reducing gingival signs of inflammation (19). The use of CHX on the day of surgery and several days after, has shown to prevent or reduce the occurrence of alveolitis after third molar extractions (20,21). CHX is also used as a pre-rinse to reduce aerosolization of microbes and viruses during dental procedures. In a recent study, CHX mouthwash has shown to be effective in reducing the SARS-CoV-2 viral load in saliva for a short-term period, two hours after using 15ml 0.12% CHX once (22).

Orthodontic patients have a high prevalence of gingivitis due to the presence of brackets, bands and other accessories that facilitate biofilm and calculus formation (23). Data indicate that the use of CHX mouthwash at 0.12% concentration in a short-term period of 3 months, in addition to regular oral hygiene habits, is effective in reducing plaque and gingivitis in adolescents undergoing orthodontic treatment (24). Also, a mouthwash of CHX at 0.2% and toothbrushing has proven to reduce significantly biofilm and gingivitis levels in adolescents wearing brackets (25,26). The use of CHX mouthwashes at 0.2% in patients with fixed orthodontic appliances led to a significant reduction in the level of *Streptococcus mutans* (27). Nonetheless, because of its adverse effects after continuous use, CHX at both concentrations 0.12% and 0.2% should not be indicated for long-term periods, and only should be considered for treating acute gingival inflammation.

CHX is normally used in concentrations ranging from 0.12% and 0.2%. Both concentrations have been reported as safe, and have a low level of tissue toxicity. Allergic reactions to CHX are not a common adverse event reported in the general population. Even though it has been pointed out as an efficacious compound with antibacterial activities, it use has been associated with staining of teeth and oral mucosa, and in some cases may be related with dysgeusia. Therefore, mouthwashes with concentrations of CHX at 0.12% and 0.2% are both recommended for short-term use. Lower doses of CHX (0.06%) provide a comparable effect in preventing biofilm formation and control of gingivitis when compared to formulations with higher CHX concentrations. The type of action is dose dependent, for instance, lower concentrations of CHX have been reported to have bacteriostatic properties, and higher concentrations a bactericidal effect (1). A clinical study examined the dental plaque and gingivitis inhibitory effects of low-dose 0.06% CHX preparations in comparison with CHX at 0.1%, an amine fluoride/stannous fluoride (ASF) solution and a water control as an adjunct to the daily mechanical oral-hygiene measures (28). After three months, participants using both concentrations of CHX had less plaque accumulation, suggesting that if maintaining clinical health is the goal, CHX at 0.06% is a good alternative to CHX at 0.1% since patients exhibit less teeth staining (29).

Even though CHX was introduced to the dental market almost seven decades ago, few studies have evaluated the antimicrobial and antifungal capacity of different concentrations of CHX mouthwashes. Therefore, the aim of this study, was to evaluate *in vitro*, the antibacterial and antifungal capacity of three commercially available mouthwashes, with different concentrations of CHX, 0.12%, 0.06%, and 0.03%.

MATERIALS AND METHODS

To test the antibacterial and antifungal capacity of three available mouthwashes with

different concentrations of CHX, three molecules were selected from the same company (Stein Co, Cartago, Costa Rica). All the samples were blinded for the experimental operator, who only identified the solutions by its color. The commercial mouthwashes tested are shown in table 1.

Table 1. Commercial mouthwashes evaluated.

Commercial name	Chlorhexidine gluconate concentration	Additional active compounds
Clorexil Profesional®	0,12%	None
Clorexil Gingival®	0,06%	0,12% Zinc + 0,05% Sodium Fluoride
Clorexil Desensibilizante®	0,03%	0,2% Sodium Fluoride + 5% Potassium nitrate

The experimental method selected was the Kirby-Bauer method to evaluate the antibacterial effect of each compound by measuring the inhibitory effect of the bacteria or fungal strain exposed to the antiseptic solution. This method was employed selecting 4 different strains of ATCC cultures. Table 2 summarize the strains and the antibiotic or antifungal agent used as control.

Table 2. Strains and antibiotic/antifungal controls.

ATCC Strain	Antibiotic / Antifungal disc
Staphylococcus aureus subsp. Aureus ATCC® 25923™	Amoxicillin + Clavulonic Acid 30mcg
Enterococcus faecalis ATCC® 29212™	Ceftriaxone 30mcg
Escherichia coli ACCT® 8739™	
Candida albicans ATCC® 90028™	Voriconazol 1mcg

Bacteria strains were seeded in individual blood agar plates, while Candida albicans ATCC® 90028™ (C. albicans) was seeded in Sabouraud dextrose agar (SDA). All strains were cultured for 24 hours. From each bacterial strains, two pure colonies were seeded in tryptic soy broth: and for *C. albicans* in yeast extract peptone dextrose agar. Broths were homogenize in continuous stirring under 100rpm, at 37ºC. Three consecutive washes with phosphate buffer solution (PBS) were performed, and colonies were adjust to a 0,5 standard McFarland concentration scale. Bacteria strains were seeded in Mueller-Hinton Agar; and C. Albicans in Mueller-Hinton with Methylene Blue and dextrose.

The antiseptic discs were prepared adding 20µL of each solution to standard clean paper discs, while standard antibiotic/antifungal discs were selected according to each strain. Each disc was placed 5 minutes after the strains were seeded, and each one kept a minimal distance of 20mm between each other. All samples were prepared by triplicate. After all the discs were placed, Petri dishes were placed in an incubator at 37ºC for 24 hours. The antimicrobial effectiveness was evaluated by measuring the zones of inhibition, using a millimetric rulers that must pass right in the middle of the disc (with a standard measure of 6mm).

STATISTICAL ANALYSIS

The statistical analysis was done using R Software version 3,5. Shapiro-Wilk's test was used to evaluate the normality of the data, and the Levene´s test to evaluate the homogeneity of variances. The differences between the zones of inhibition was evaluated by one-way ANOVA. Statistical significance was determined at $p<0.05$.

RESULTS

For the *Staphylococcus aureus subsp. Aureus* ATCC® 25923™ (*S. aureus*) group (Figure 2) the inhibition zone between the groups showed statistical difference ($F=399,6$; $p<0,001$). Multiple comparison was evaluated with a Tukey post hoc test, showing that the antibiotic inhibition zone of the antibiotic disc was statistically diffe-

rent (p<0.001) when compared with the three mouthwashes. All the mouthwashes were different between each other, showing an inhibition zone proportional to the concentration of the CHX.

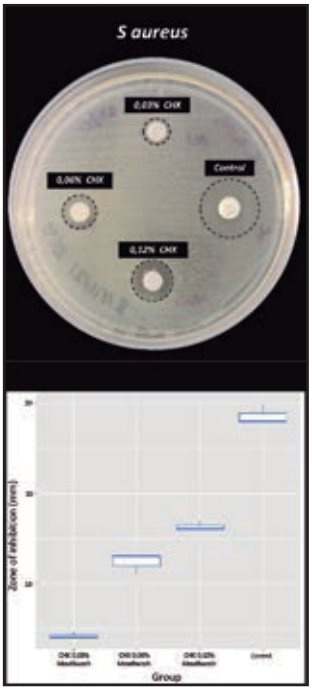

Figure 2. Box plot analysis and representative image of microorganism inhibition experiments for *S. aureus* group.

Enterococcus faecalis ATCC® 29212™ (*E. faecalis*) group showed significant statistical differences between the inhibition zones of all samples (F=73,54; p<0,001) (Figure 3). Multiple comparison was evaluated with a Tukey post hoc test, showing that the antibiotic inhibition zone of the antibiotic disc showed statistically significant differences (p<0.001). Mouthwashes containing 0,12% and 0,06% CHX didn't showed significant statistical differences, but both groups were different than the 0,03% CHX mouthwash.

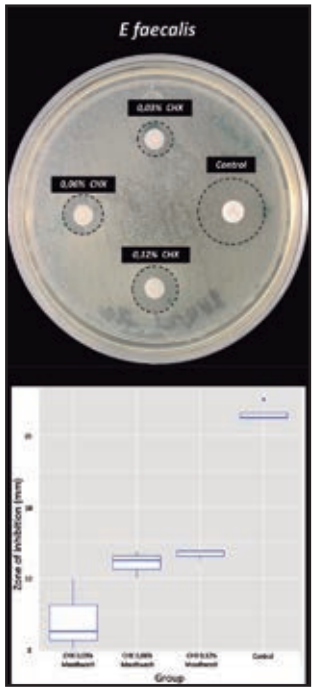

Figure 3. Box plot analysis and representative image of microorganism inhibition experiments for *E. faecalis* group.

Escherichia coli ACCT® 8739™ (*E. coli*) group showed significant statistical differences between the inhibition zones of all samples (F=46.28; p<0,001) (Figure 4). Post hoc Tukey analysis showed significant statistical differences for the antibiotic disc, superior than all the mouthwashes, with no differences between each other (p>0.05).

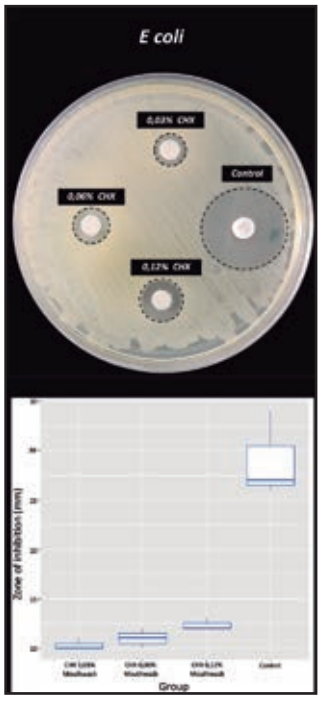

Figure 4. Box plot analysis and representative image of microorganisms inhibition experiments for *E. coli* group.

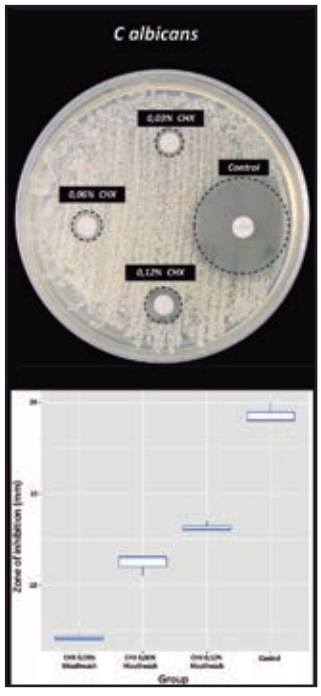

Figure 5. Box plot analysis and representative image of microorganisms inhibition experiments for *C. albicans* group.

Finally, antifungal effect in the *C. albicans* group showed also significant statistical differences between the inhibition zones of all samples ($F=1041$; $p<0,001$) (Figure 5). Post hoc Tukey analysis showed significant statistical differences within the groups. The inhibition zone of the antifungal disc was superior to all mouthwashes ($p<0.001$); followed by 0,12% CHX mouthwash that showed significant statistical differences ($p<0.05$). 0,06% and 0,03% CHX mouthwashes didn´t show significant statistical differences.

DISCUSSION

In this study, the antibacterial and antifungal capacity of three commercially available mouthwashes with different concentrations of CHX (0.12%, 0.06%, and 0.03% respectively) was evaluated in vitro. All samples showed some degree of antibacterial and antifungal effect.

The anti-staphylococcal activity of CHX mouthwashes at concentrations of 0.2% and 0.12%

had been observed previously in other studies (30, 31, 32, 33). In the present investigation, all the mouthwashes inhibited *S. aureus* growth *in vitro*. The three different concentrations showed an inhibition zone proportional to the concentration of CHX. Our results suggest that lower doses of CHX are useful as part of decontamination regimens to decolonize presence of staphylococcal activity in the oral cavity and oropharynx. Isolates of S. *aureus* have been obtained in subgingival and supragingival plaque of patients with periodontitis and/or periimplantitis. This is relevant, since the reduction in the number of *S. aureus* in the oral cavity, prior to surgical procedures, has been associated with a lower incidence of infective endocarditis and postoperative complications (32) in patients with respiratory infections.

The antimicrobial effect of 2% chlorhexidine against *E. faecalis* has been vastly reported in literature. In this study, the three concentrations of CHX mouthwashes analyzed were effective against *E. faecalis*, showing a lower effect than the control. Concentrations of 0,12% and 0,06% didn´t showed statistical differences, but both were statistically different when compared to 0,03% concentration. *E. faecalis* is a component of the normal oral microbiota. It is of interest in endodontic research, since it is one of the species mainly involved in failures of root canal treatment (34,35). *E. faecalis* can exhibit resistance against common disinfectants and endodontic irrigants (36,37). Further research must evaluate the clinical importance of using regularly low concentrations of CHX, against the *E. faecalis* community in oral cavity.

E. coli is a gram-negative bacteria, commonly found in the gut of humans, and most strains do not cause human disease. However, some strains produce toxins and other virulence factors, such as lipopolysaccharide, that may produce food poisoning if people swallow *E. coli* by eating contaminated foods, by drinking contaminated water, or by hand to mouth contact. The presence of *E. coli* has also been reported in subgingival biofilm of patients with periodontitis, and a significant higher prevalence was observed in diseased sites of periodontal patients compared to periodontally healthy subjects (38). Case reports and *in vitro* studies have associated gram-negative enteric rods to early dental implant failure and peri-implantitis (39,40,41). Our results indicate that the three concentrations of CHX mouthwashes displayed a similar inhibition zone to *E. coli*. It has to be considered that only a single strain of *E. coli* was used in this experiment. Therefore, this may pose a limitation in the interpretation of the results since there can be great genetic variation with different strains of the same specie.

The antimicrobial activity of chlorhexidine against *C. albicans* and other common non-albicans yeast species has been documented and advocate the use of CHX gluconate as an adjunct in the management of oral candidiasis (42,43,44). CHX at a concentration of 0.2% has shown a significant antifungal activity comparable to ketoconazole (45). Even a brief exposure to subtherapeutic concentrations of chlorhexidine (0.00125, 0.0025 and 0.005%) for thirty minutes, modulates germ tube formation of *C. albicans* isolates and may suppresses pathogenicity (45). In our study, the inhibition zone of 0,12% CHX mouthwash was superior to lower concentrations, 0,06% and 0,03%. However, all showed antifungal capacity. Such result is particularly important, considering vulnerable populations to fungal oral infections, such as removable prothesis users or immunocompromised patients. Low dose chlorhexidine mouthwash employed in these patients routinely may show important clinical benefits.

A meta-analysis that included publications evaluating CHX's efficacy against bacterial plaque and gingivitis for six months, demonstrated superiority in those patients who included CHX mouthwashes in their oral hygiene routine (46).

CHX at a concentration of 0.12% demonstrated statistically superior efficacy controlling dentobacterial plaque in all the publications evaluated (46). In cases of generalized gingivitis, CHX mouthwashes have shown greater efficacy than the use of gels (47). CHX rinsing protocols should be the first choice for those patients where daily oral hygiene is difficult (47). Rinsing for 60 seconds 2 times a day with 10mL of chlorhexidine can inhibit the growth of dentobacterial plaque by 60%; and reduce gingivitis by 50-80% (4).

Even though we provide *in vitro* results, our findings are of relevance since all the species used in our experiment are microorganisms that may be present in dental plaque. Most studies on CHX's control of dental plaque and clinical indices do not show differences between concentrations of 0.12% and 0.2%; however, at lower doses, fewer adverse effects have been reported. In our study, the three concentrations of CHX had antibacterial and antifungal capacity. Low dose regimens could therefore reduce side effects, such as tooth discoloration, taste disturbance, stain and mucosal erosions, produced by CHX, but maintain reasonable antibacterial and antifungal efficacy.

Although this research offers important evidence of the antiseptic effect of CHX mouthwashes, important limitations and perspectives must be discussed before considering clinical extrapolations. First, this *in vitro* study may not reflect the clinical scenario in humans, considering the existence of multispecies biofilm in both periodontal and dental tissues, so confirmatory clinical data will be needed. Second, the use of ATCC strains may behave different from planktonic bacteria obtained from different populations, thus further *ex vivo* studies using bacteria obtained from human patients is suggested. Finally, a comparison of the observed effect on single cultured bacteria in Petri dishes vs biofilm *in vitro* models will help to confirm the validity of these results.

CONCLUSIONS

The low dose and therapeutic concentrations of chlorhexidine in the mouthwashes showed a different degree of inhibitory effect against the bacterial and fungal strains tested, exhibiting a concentration related behavior in most of the cases.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization and design: DCB, VEV.
Literature review: DCB, KRC.
Methodology and validation: DCB, VEV.
Formal analysis: DCB, KRC, VEV.
Investigation and data collection: VEV.
Resources: DCB, VEV.
Data analysis and interpretation: DCB, KRC, VEV.
Writing-original draft preparation: DCB, KRC.
Writing-review and editing: DCB, KRC, VEV.
Supervision: DCB, KRC, VEV.
Project administration: DCB, KRC, VEV.
Funding acquisition: DCB, KRC, VEV.

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