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RESEARCH ARTICLE OPEN ACCESS

In vivo Evaluation of the Antagonistic Effect of *Lactobacillus acidophilus* against *Propionobacterium acnes* in the Treatment of Acne

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Abstract

Probiotics are able to inhibit certain pathogens, their use to fight acne caused by the proliferation of *Propionibacterium acnes*, would be an alternative in topical bacteriotherapy. There are studies of *in vitro* antagonism[1]. This study i*n vivo* the antagonistic capacity of *Lactobacillus acidophilus* against *Propionibacterium acnes* in young people from 15 to 20 years old, with previous diagnosis of type II acne. First the evaluation of the *in vitro* antagonistic effect of *Lactobacillus acidophilus* against *Propionibacterium acnes* was performed. The measurement of the inhibition halos determined the concentration with antagonist effect 1x10^8 cfu/ml, with this concentration four formulations were tested, of these four only one had an *in vitro* effect; this formulation was subjected to a test of irritability (Mexican standard Nom-039-SSA1-1993) IPC = 0.19 defines the formula as "well tolerated", the study of cosmetic activity *in vivo* was performed with a non-invasive method of exploration , using the Visiopor PP 34N® bioengineering instrument as a result, a 48-hour decrease in average porphyrins of 78.3% was obtained in the total number of treated individuals, which implies a decrease in the population of *P. acnes*, the acne-causing agent.

Keywords: Acne, antagonist effect, *Lactobacillus acidophilus, Propionibacterium acnes.*

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INTRODUCTION

Lactic acid of *Streptococcus thermophilus*, a bacterium found in yoghurts has specific benefits in the skin, because it can increase the production of ceramide when applied to the skin for 7 days[2]. It has been reported that this bacterium possesses mainly some of the sphingolipids of this ceramide, especially phytosphingosine (PS), which provide antimicrobial activity against *P. acnes*, which would be of great interest for the treatment of acne[3]. Other researches carried out have shown that numerous probiotic bacteria can provide antimicrobial *in vitro* activity against *P. acnes*, as is the case of *Enterococcus faecalis* SL-5 which when applied topically through a lotion for 8 weeks reduces inflammatory lesions caused by *P. acnes* by more than 50% compared to placebo[4]. There is no detailed information on the antagonistic activity of *Lactobacillus acidophilus* against *P. acnes*, however, there is an *in vitro* study of the ability of probiotic bacteria to inhibit acne produced by *P. acnes*, such as that performed by AI-Ghazzewi and Tester (2010), who studied four strains of Lactobacilli (mostly of human origin), *Lactobacillus casei* ssp. *casei* NCFB161, *Lactobacillus acidophilus* NCFB 7148, *Lactobacillus plantarum* DSM 12028, *Lactobacillus gasseri* NCFB 2233 and a Lactococcus strain (*Lactococcus lactis* ssp. *lactis* NCIMB 6681) which showed a significant inhibition in the growth of *P. acnes* NCTC737, but inhibition concentrations were not defined, and were not tested *in vivo*[5].

Once the antagonistic activity is demonstrated, it is necessary to incorporate the probiotic into a cosmetic formulation to prove the antagonistic capacity of *Lactobacillus acidophilus*; the efficacy of the formula will be verified by the reduction of porphyrins in the treated skin, an aspect directly related to the decrease in the population of *P. acnes* in individuals, a result that allows promoting the use of probiotic as an alternative In the treatment of acne.

MATERIALS AND METHODS

Biological Material

Lactobacillus acidophilus ATCC® 4356 (probiotic lactic bacteria)

Propionibacterium acnes ATCC® 11827

Antagonism tests

Pathogenic bacterium inoculum (*P. acnes* ATCC® 11827) (100µl) was inoculated in Mann Rogosa Sharp agar (MRS, BioCen); Whatmann discs of 6 mm diameter were placed in in the dry agar antibiogram type, on each one were added 10 ml of three different concentrations of *L. acidophilus* ATCC® 4356. The second antagonism test was carried out with the cosmetic formulations, in which the concentration with the best antagonism of the first stage was incorporated. The boxes were placed in inverted position inside the anaerobic incubator at 37°C in 5% of CO_2 for 72 hours. This test was determined as positive when grow was presented around the antibiogram disk.

Elaboration of the Cosmetic Formula

Cosmetic form is defined as a lotion with the least number of ingredients to evaluate the efficacy of the element considered as active in the formulation 1 x 10^8 cfu/ml of *Lactobacillus acidophilus*, the other Ingredients are specified in table 1.

Table 1. Ingredients of the cosmetic lotion

Ingredient (INCI)	Role	Use percentage
Glycerin	Moisturizer agent	10 -30%
Cellulose gum	Emulgent agent	0,25 – 1%
Distilled water	Vehicle	e.c.f

*e.c.f. = enough quantity for

Four formulas were tested based on an experimental design that combined the concentrations of the two excipients of the formula; i 2x2 experimental factorial design was done.

Evaluation of the dermal safety of the lotion (irritability test)

All *in vivo* tests were carried out considering the ethical criteria of the research in human beings, and from the selection process all the volunteers were informed about: risk–benefit, confidentiality and informed consent.

The procedure for applying the patch Test was based on the official Mexican standard Nom-039-SSA1-1993 with human induction patch tests, bringing the cosmetic sample into contact with the person's skin to measure dermal irritation and sensitivity; the results were valued according to a numerical scale that is averaged, the average

represents the irritation average index (I.A.I) which is an useful index to assess the damage that can present the products applied on the skin[7].

***In vivo* evaluation: efficiency of the cosmetic formula**

A non-invasive methodology was performed by using a bioengineering team Visiopor PP34N® which quantifies the number of porphyrins per cm^2, and a mathematical analysis that determines the reduction of porphyrins directly related to the decrease of *P. acnes*. The selection of the voluntary individuals was made based on the inclusion and exclusion criteria, the dermatological clinical evaluation that identified twenty adolescents diagnosed with acne type II; the volunteers filled out a file with their personal data and clinical background and the informed consent letter.

Ethical considerations in the *in vivo* study

The investigation complied with all the parameters established in the Organic Health Law of Republic of Ecuador, in which is established in the Art. 208 that any scientific technological research in health must be subjected to bioethical and rights principles, prior informed and written consent and respecting confidentiality[8]. In terms of respecting these bioethical principles, the research process was based on three principles: autonomy, beneficence and not maleficence. Being understood autonomy as the recognition of people to choose rationally and to govern their own body according to their own interest to the extent of their possibilities; in this sense the decisions are around the capacities of the subject under study and not on the investigator[9]; based on this principle each voluntary subject of study was timely and pedagogically informed about the procedure to follow, its implications in the short, medium and long term, the benefits and risks of the procedure and on the possibility of leaving at any time of the study, to ensure this principle was handled the "Confidentiality Agreements" and "informed consent". The beneficence is understood as the obligation that the investigator has to act for the benefit of the subject under investigation, helping the individual to promote his/her legitimate interests[10]. To fulfill both the principle of beneficence and non-maleficence, the research was managed with a "research protocol" which raised the risks involved in the study, risks that are few by being a non-invasive therapy, on the contrary, therapy with a lot of benefits for the volunteer subjects who were presented this alternative to improve their skin pathology.

RESULTS AND DISCUSSION

***In vitro* antagonism test**

The results of the antagonism test are shown in Table 2 as well as the averages of the inhibition halos and the inhibition area or free area.

Sterile water was used as negative white and an antibiotic (tetracycline) as a positive white.

Table 2. Inhibition halos of *Lactobacillus acidophilus* ATCC® 4356 against *Propionibacterium acnes* ATCC®11827

Lactobacillus acidophilus (cfu/ml) conce.	Average of inhibition halos (mm)	Inhibition area (inhibition halo–antibiogram disc) (mm)
$1x10^8$	9.8	3.8
$1x10^7$	6.3	0.3
$1x10^6$	6	0
BP (positive white)	37.4	31.4
BN (negative white)	6	0

Table 3. Cosmetic formulas (lotions) with *L. acidophilus*

Components	Lotion A	Lotion B	Lotion C	Lotion D
L. acidophilus	1 $x10^8$ cfu/ml	1 $x10^8$ cfu/ml	1 $x10^8$ cfu/ml	1 $x10^8$ cfu/ml
Glycerin	10%	10%	30%	30%
Cellulose gum	0.25%	1%	0.25%	1%
Distilled water	e.c.f.	e.c.f.	e.c.f.	e.c.f.

*cfu= colony forming units

According to the results, *Lactobacillus acidophilus* showed certain tendency to inhibit the pathogen from a concentration of 1 x 10^7 cfu/ml; however, the concentration that presented higher inhibitory activity was 1 x 10^8 cfu/ml, with an inhibition area higher than 1 mm around the disc, which was determined as a positive antagonistic effect.

The antagonistic effect obtained against *P. acnes* (Gram-positive bacteria) was less than the expected with respect to the inhibition obtained in various studies against other pathogenic microorganisms, this may be because lactic bacteria exert more antagonistic activity against gram-negative bacteria compared to gram-positive bacteria, mainly because gram-negative bacteria have an external membrane containing phospholipids, lipopolysaccharides and proteins that traverse the wall in all its thickness, delimiting hydrophilic pores that allow the passage of substances with low molecular weight so the sensitivity is different[11].

Statistical analysis of inhibition halos generated by *Lactobacillus acidophilus* ATCC® 4356

Fig. 1 shows the concentration mean of 1 x 10^8, which is 9.8, and the interquartile range is 0.7; the concentration median of the 1 x 10^7 is 6.35, and the Interquartile range is 0.52;

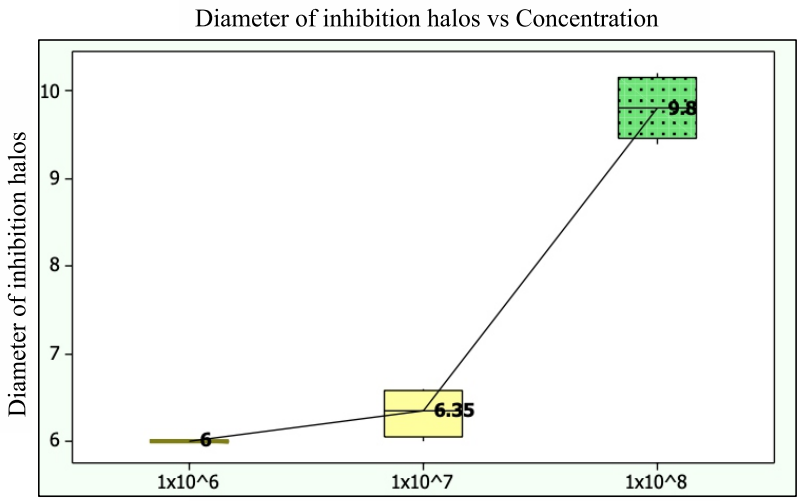

Fig. 1. Box plot of inhibition halos diameter vs the concentration of *Lactobacillus acidophilus* carried out in MINITAB 1.6.

the concentration mean of 1 x 10^6 is 6, and the Interquartile range is 0.

The tendency line increased from the concentration 1 x 10^6 to the concentration 1 x 10^8, indicating that the lowest the concentration of *L. acidophilus* is the diameter of the inhibition halo.

To determine which of the concentrations causes the difference of the halos, a post-test was carried out in which the means of all the possible concentration pairs (Kruskal-Wallis All-Pairwise Comparisons test) were obtained, from which the following results were obtained and are shown in Figure 2.

According to the results presented in Figure 2, there are two groups of concentrations which are statistically related to each other. These groups are: Group A: 1x10^8 cfu/ml, 1x10^7 cfu/ml and group B: 1x10^7 cfu/ml, 1x10^6 cfu/ml.

The concentration that that stands out from the two groups that were formed is 1x10^8 cfu/ml, because it had a higher mean average (10500). Therefore, it is concluded that it presents the best inhibition halos.

Elaboration of cosmetic formulas and *in vitro* antagonism tests

According to the 2x2 experimental design, four formulas were generated and are presented on Table 3; these formulas were done to carry out the *in vitro* antagonism test and to ensure that the incorporation of excipients in the formula not affect the antagonistic activity of *L. acidophilus.*

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Kruskal-Wallis All-Pairwise Comparisons Test

Variable    Mean  Homogeneous Groups
V001      10.500  A
V002      6.0000  AB
V003      3.0000   B

Alpha              0.05
Critical Z Value  2.394     Critical Value for Comparison
6.1035
There are 2 groups (A and B) in which the means
are not significantly different from one another.
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Fig. 2. Kruskal-Wallis All-Pairwise Comparisons Test of *Lactobacillus acidophilus* ATCC ® 4356 concentrations against *Propionibacterium acnes* ATCC® 11827.

The inhibition halos of lotions formulated with *L. acidophilus* are presented in Table 4, formulas A, B, C or D without *L. acidophilus* are considered as a negative white, that is, only the excipients of each formulation.

Table 4. Mean of inhibition halos (mm) of cosmetic formulas with *L. acidophilus*

Formula	Average of inhibition halos (mm)
Lotion A	9.6
Lotion B	8.5
Lotion C	8.2
Lotion D	6.3
Negative White (A, B, C, D)	6.0

Statistical analysis of inhibition halos of cosmetic formulations

In figure 3 is determined that the median diameter of the inhibition halo was higher for lotion A (9.6), this lotion also demonstrated the least variability, with an interquartile range of 0.37. In addition, the distribution had a negative asymmetry. Lotions B and C had median diameters of similar inhibition halos (8.5 and 8.2, respectively). In addition, lotion C also showed the highest variability, with a median interquartile range of 0.85. Halos diameter of lotion inhibition D was only 6.25 with an interquartile range of 0.65. The BN median of each of the lotions was 6, indicating there is no type of inhibition.

Kruskal-Wallis (H) statistic value was 13.0336, and the probability value associated with

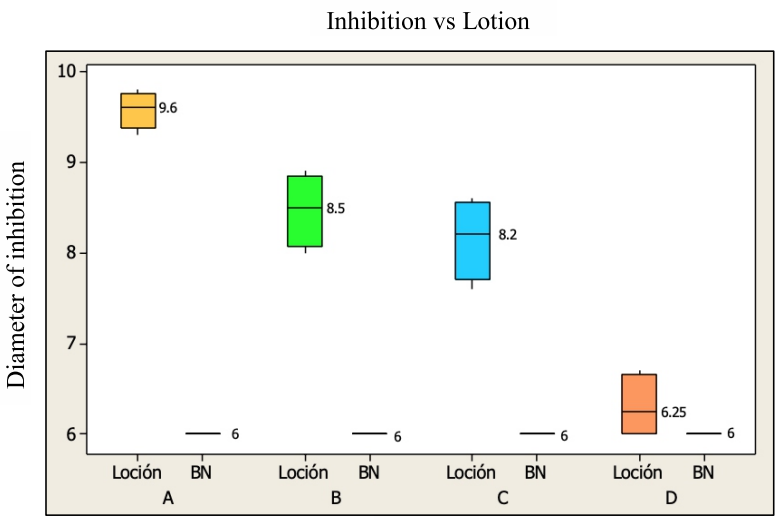
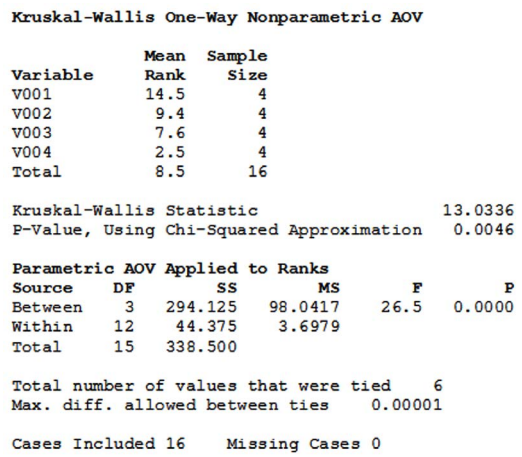


Fig. 3. Box plot of inhibition diameter vs experimental lotions, made in MINITAB 1.6.



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Kruskal-Wallis One-Way Nonparametric AOV

            Mean   Sample
Variable    Rank     Size
V001        14.5        4
V002         9.4        4
V003         7.6        4
V004         2.5        4
Total        8.5       16

Kruskal-Wallis Statistic                  13.0336
P-Value, Using Chi-Squared Approximation   0.0046

Parametric AOV Applied to Ranks
Source    DF        SS         MS        F         P
Between    3   294.125    98.0417    26.5    0.0000
Within    12    44.375     3.6979
Total     15   338.500

Total number of values that were tied    6
Max. diff. allowed between ties    0.00001

Cases Included 16    Missing Cases 0
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Fig. 4. Kruskal-Wallis Test for four lotions with *Lactobacillus acidophilus* ATCC® 4356 against *Propionibacterium acnes* ATCC® 11827.

the statistic (P) was 0.0046. The probability value associated with the statistic (P) was less than the chosen Alpha (0.05); for this reason, the alternative hypothesis is accepted and it is concluded that at least one of the lotions generated an inhibition halo different from the others.

In order to determine the lotion generated different inhibition halos, a posteriori test was performed in which the medians of all possible pairs of lotions (Kruskal-Wallis All-Pairwise Comparisons test) were compared, from which were obtained the following results, which are presented in Figure 5.

There are two groups of lotions which are statistically similar. These groups are: Group A: lotion A, lotion B, lotion C and Group B: lotion B, lotion C, lotion D. When comparing the groups, it was observed that the lotion with the highest medium range is lotion A, with a median range of 14,500, considering the one with most antagonistic effect; so it was used in the study of cosmetic activity *in vivo*.

Evaluation of the dermal safety of the lotion (irritability test)

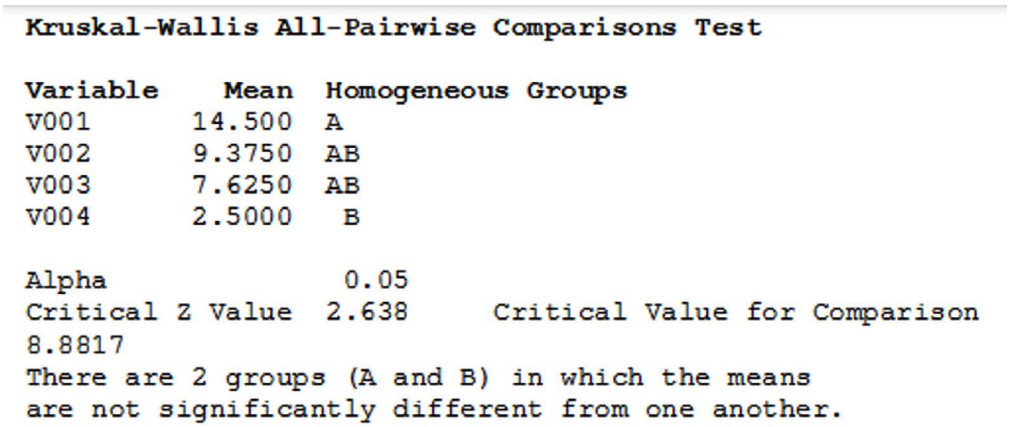
The I.A.I. of lotion A was 0.19, the standard identifies that if the I.A.I is between > 0 and ≤ 0.5 it qualifies as a "well tolerated" product that can be safely used, and in the specific case of this investigation it was suitable to continue with the efficacy study *in vivo*.

***In vivo* evaluation: efficacy of the cosmetic formula**

After the timely application of lotion A in acne lesions for 2 consecutive days, the amount of porphyrins was quantified at the beginning, 24 hours later and at 48 hours in volunteered adolescents using the Visiopor PP34N ® team.

Table 5 shows that there was a decrease in porphyrins since the first application of the formulated lotion; a maximum decrease of 100% was obtained 48 hours after the application, and a minimum of 45.5%, and a total average of 78.3%, indicating that lotion A had antagonistic effect against *P. acnes in vivo*.

As shown in Figure 6, all adolescents in the study showed a porphyrins reduction of more than 40%. Eight out of the 20 individuals showed higher percentage decrease in a range of 90% - 100%.



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Kruskal-Wallis All-Pairwise Comparisons Test

Variable    Mean  Homogeneous Groups
V001      14.500  A
V002      9.3750  AB
V003      7.6250  AB
V004      2.5000   B

Alpha              0.05
Critical Z Value   2.638     Critical Value for Comparison
8.8817
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

Fig. 5. Kruskal-Wallis All-Pairwise Comparisons Test for the four lotions with *Lactobacillus acidophilus* ATCC® 4356 against *Propionibacterium acnes* ATCC® 11827.

Table 5. Reduction percentage of porphyrins generated by *P. acnes* after the application of lotion A.

Volunteer N^0	Initial evaluation (N^{0}of porphyrins)	Evaluation 24 hours later (N^0 of porphyrins)	Final evaluation 48 hours later (N^0 of porphyrins)	Reduction of porphyrins	Reduction % of porphyrins
1	7	5	3	4	57.1
2	2	0	0	2	100
3	8	4	2	6	75
4	11	8	3	8	72.7
5	22	14	12	10	45.5
6	8	5	3	5	62.5
7	4	3	2	2	50
8	7	4	0	7	100
9	8	4	1	7	87.5
10	16	4	2	14	87.5
11	3	2	1	2	66.7
12	1	0	0	1	100
13	2	1	1	1	50
14	4	4	2	2	50
15	21	14	7	14	66.7
16	5	3	0	5	100
17	13	8	0	13	100
18	1	1	0	1	100
19	1	0	0	1	100
20	21	16	1	20	100

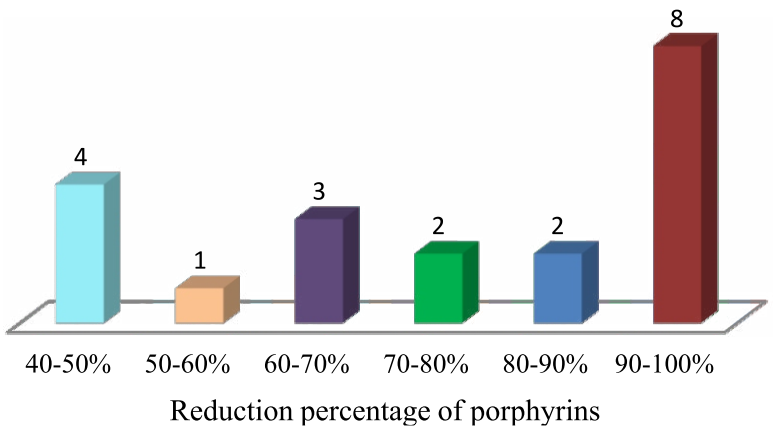

Fig. 6. Number of adolescents vs. reduction percentage of Porphyrins.

CONCLUSIONS

It is concluded that at a higher concentration of *L. acidophilus* higher *in vitro* antagonistic capacity compared to *P. acnes*; a concentration of 1 x 10^8 cfu/ml achieved higher inhibition than a concentration of 1 x 10^6 cfu/ml. From the four lotions evaluated (A, B, C, D) the one that presented higher *in vitro* antagonistic activity compared to *P. acnes* was lotion A. The irritability study determined the formula as a well-tolerated product with an I.A.I equal to 0.19 that qualifies it as a skin-safe product. The *in vivo* study of cosmetic activity showed the antagonistic activity against *P. acnes in vivo*, obtaining a total reduction average of porphyrins of 78.3%, which implies a decrease in the population of *P. acnes* and an effective alternative for treating acne.

ACKNOWLEDGMENTS

The authors thank all the individuals who participated in the *in vivo* studies in the assessment of the dermal safety of the lotion, and in the i*n vivo* effectiveness evaluation of the cosmetic formulation, because these individuals made this research possible.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

TM conducted formulation studies by establishing *in vivo* evaluation protocols, also participated in data analysis and wrote the manuscript. VP conducted the microbiological trials, coordinated the *in vivo* studies and helped to write the manuscript. Dermatologist Marcela Paredes conducted the dermatological assessment of the individuals before the instrumental evaluation study. All the authors read and approved the final manuscript.

FUNDING

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DATA AVAILABILITY

The study data was recorded in Visiopor PP 34N software, Courage Khazaka Electronic, using codes that protect the identity of participating volunteer individuals.

ETHICS STATEMENT

All the research protocols were reviewed by the research committee of Universidad Polit 'cnica Salesiana, checking that they follow the national and international codes, oriented and justified within the scientific area, with non-invasive procedures that guarantee minimal risk to participants and protect the privacy and confidentiality of participants in the processes of data collection, handling and storage.

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