


	Study report on the efficacy of BED Pro-biotic on the presence of DERP1 allergen in house dust, Perilis Trading EOOD	
	BULGARIAN ACADEMY OF SCIENCES The Stephan Angeloff Institute of Microbiology Associated with Institut Pasteur	Acad. G. Bonchev Str 26, 1113 Sofia Tel. (+359 2) 979 31 57; Fax: (+359 2) 870 01 09 e-mail: micb@microbio.bas.bg



I TITLE PAGE

Test Facility	Assignor
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<p>Evaluation of the efficacy of BED Pro-biotic on the presence of DERP1 allergen in house dust</p>	

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3 SUMMARY

The following probiotic product of Perilis Trading EOOD

- BED Pro-biotic, van der Schoot technology – probiotic neutralizer for mites and allergens

was investigated for inhibitory effect on the presence of European house dust mite *Dermatophagoides pteronyssinus*. The test was conducted in ten premises, of which two were laboratory and eight were households. DERP1 is the main allergen of house dust mite *Dermatophagoides pteronyssinus*, which has routinely been detected in residential dust. An assay for the presence of DERP1 antigen in the monitored sites was performed after administration of the product within one month. The product's application was in accordance with the manufacturer's instructions. Samples were collected prior to product administration and twice thereafter – at the end of the second and fourth weeks of the beginning of the test period. Extraction of the antigen was performed according to protocols published in the scientific literature, and the antigen was demonstrated by an ELISA immunoassay for DERP1 antigen.

The result obtained showed that the product tested effectively reduced and eliminated the presence of European house dust mite *Dermatophagoides pteronyssinus*. The quantitative data from the analysis are available in the attached detailed scientific report. This result confers only to the tested three products and any extrapolation or transfer to other products is responsibility of Perilis Trading EOOD.

4 LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

Test sample: product to be tested for antimicrobial activity

DERP1: allergen identified in *Dermatophagoides pteronyssinus*

ELISA: Enzyme-linked Immunosorbent Assay



PBS: Phosphate Buffer Saline

5 INVESTIGATORS AND STUDY ADMINISTRATIVE STRUCTURE

5.1 Investigators

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Assist. Prof. Maya M. Zaharieva, PhD

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5.2 Administrative structure of the study



Figure 1. Administrative structure of the study

6 INTRODUCTION

Allergic diseases represent major health burden. An allergic reaction is characterized by a disrupted Thelper 1/T-helper 2 balance toward a preferential allergen specifically induced TH2 cytokine profile, causing allergic inflammation [1]. House dust mites are the source of 21 allergens identified so far. The major allergens of the common dust mite (*Dermatophagoides pteronyssinus*) are DERP1 and DERP2. Exposure to these allergens is associated with allergic symptoms and asthma in sensitised people [2]. Probiotic bacteria have various beneficial effects in many pathologic situations. Studies have shown that the probiotic bacteria present in the intestinal microflora play a role in the TH1/TH2 balance and its modulation can promote the control of infectious and immune processes [1]. There are also reports on the potential of probiotic bacteria to inhibit *Dermatophagoides pteronyssinus* stimulated secretion of Th-2 cytokines and enhance the stimulation of IFN- γ [3]. Probiotics have been used for centuries in fermented dairy products. However, the potential applications of probiotics in nondairy food products, human household and agriculture have not received formal recognition. In recent times, there has been an increased interest to food and agricultural applications of probiotics, the selection of new probiotic strains and the development of new applications has gained much importance [4]. Recently, *Bacillus* bacteria are attracting increasingly the attention of scientists all over the world because of their beneficial role in the environment and host organisms. They consistently enter the gastrointestinal and respiratory tract of humans and animals with food,

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water, air because they are ubiquitous in nature, and thus represent a part of the normal gut and foods' microflora. Strains with unique activity can be isolated among *Bacillus* bacteria [4]. Bacilli are stable during processing and storage of food, pharmaceutical and other preparations, which make them suitable candidates for health promoting formulations. *Bacillus* strains also possess biotherapeutic potential, which is connected with their ability to interact with the internal milieu of the host. Several mechanistic studies have attempted to underline the probable mechanism of action of candidate probiotic *Bacillus* strains to enhance health of the host. These mechanisms include stimulation of the immune system, synthesis of different antimicrobials, like bacteriocins and enzymes, promotion of growth of other beneficial microbes and suppression of pathogens and pathogen induced inflammatory response of intestinal mucosa [5].

The current study is focused on the evaluation of the antiallergenic activity of a probiotic product based on *Bacillus* strains and intended for use in the human life and household in terms of direct inhibition of the amount (increase, multiplication) of *Dermatophagoides pteronyssinus* in house dust. Our hypothesis for the antiallergenic potential of this product is based on published scientific evidences about the above listed mechanism of action of numerous *Bacillus* strains investigated in other scientific studies.



7 STUDY OBJECTIVES AND EVALUATED PARAMETERS

7.1 Objectives

Aim of the current study was to investigate the antiallergenic activity of the probiotic product Pro-biotic BED – probiotic neutralizer for mites and allergens (Perilis Trading EOOD, van der Schoot technology) by evaluating the presence of the allergen DERP1 in house dust after regular application of the product.

7.2 Evaluated parameters

- Quantitative colorimetric measurement of the presence of DERP1 in house dust by using DERP1 specific ELISA

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8 INVESTIGATIONAL PLAN

8.1 Description of the test sample

The tested product, delivered by the assignor was identified and described as follows:

Name: BED Pro-biotic, van der Schoot technology – probiotic neutralizer for mites and allergens

Expiry date: 12.2020

Content: Fermentative bacteria < 5 %, hypoallergenic perfume composition

Intended use: removes successfully mites and their excrement as sources of discomfort and allergies. It limits the nutrition of mites through a competitive mechanisms and release enzymes which neutralize the excrement of the mites.

Package: A white, non-transparent 500 ml plastic spray bottle.

Conditions for storage: At temperature between 5 and 35 °C.

8.2 Study design and methods

An ELISA assay was performed to evaluate the antiallergenic activity of the test sample after optimization of the extraction method:

- 1) Optimization of the extraction method
- 2) DERP1 specific ELISA assay

The study design is presented in the following diagram:

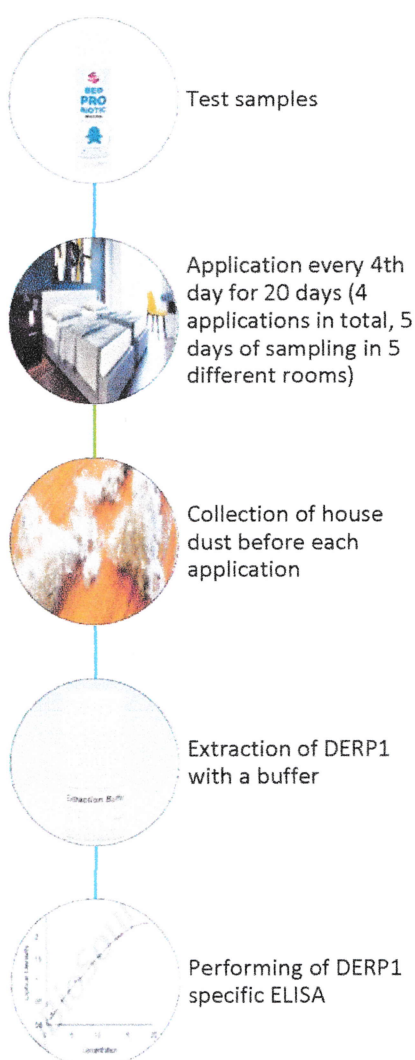




Figure 2. Study design and methods

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8.3 Study performance

8.3.1 Application of the test sample and collection of house dust

The test probiotic product was applied in five different rooms every fifth day after cleaning throughout one month. Dust samples were collected before cleaning on the 1st, 5th, 10th, 15th and 20th day in a sterile vehicle using sterile. The test was performed at the end of the Spring and beginning of the Summer in 2019.

8.3.2 Extraction of DERP1

The procedure for extracting DERP1 from reservoir dust requires optimization of the extraction buffers used. Based on literature data [2], we tested the extraction efficacy of different buffers: phosphate (pH 7.4), borate (pH 8.0), and ammonium bicarbonate (pH 8.0), all with 0.05 % Tween 20. In line with the article used, we found that all three buffers are suitable for DERP1 extraction and continued our work with the borate buffer. This decision for the borate buffer was based on a correspondence regarding the article of Prester et al. [2] with the journal editor wherein the borate buffer was claimed to exhibit twofold better efficacy in comparison to the other two buffers [6]. The borate buffer consisted of 0.11 mol L⁻¹ H₃B₃O₃, 0.044 mol L⁻¹ HCl, and 0.056 mol L⁻¹ NaOH and 0.05 % Tween 20. The extraction of the allergen from house dust was performed according to the protocol of Prester et al. [2]. Briefly, each sample was sieved through a 300 µm sieve, mixed until homogenous, and weighed. Fine dust samples were divided into two 100-µg aliquots and 2 mL of extraction solution was added to each aliquot. Extractions were done at room temperature with constant shaking on a Vortex mixer (V-1 plus, Biosan) for 2 h. After 10 min of centrifugation at 1,000xg, supernatants were stored in plastic tubes at -20 °C until analysis for DERP1 content. A total of 25 dust extracts were analysed for DERP1 content.

8.3.3 DERP1 specific ELISA

The ELISA assay was performed according to the manufacturer's instructions. Briefly, 10 µL of each sample was added to a sample well. A sample diluent was added also to each well except for the Blank well. A total of 100 µL HRP/conjugated reagent was added to each well, the plate was covered with a plate membrane, gently shaken and mix for 60 min at 37 °C. Each well was washed 5 times with 350 µL washing solution and dried. Thereafter, 50 µL chromogen solution A was added to each well followed by 50 µL chromogen solution B. Following 15 min incubation (37 °C, away from light), 50 µL Stop solution was added to each well to stop the reaction.

8.3.4 Recording of the results

The absorbance of the color product was measured on a microplate reader EL x 800 (BioTek, USA) at 450 nm wave length and saved in Excel format.

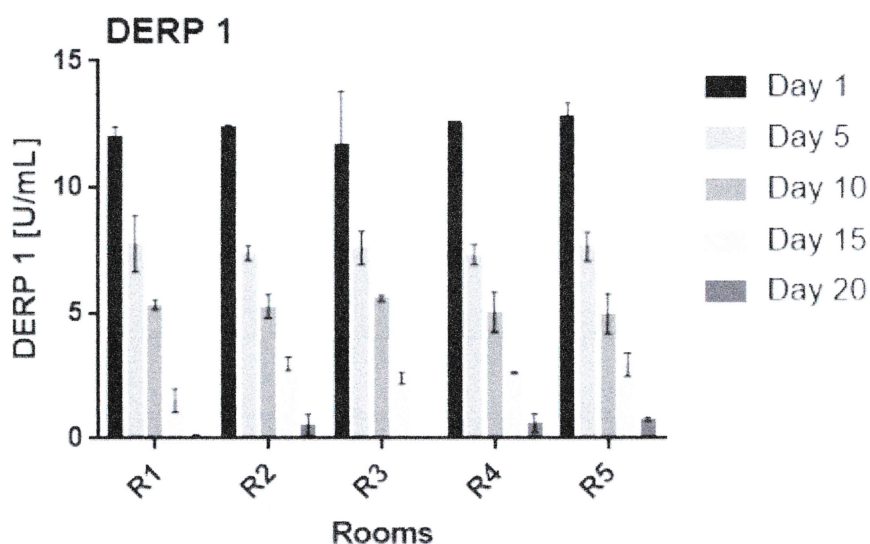
8.4 Evaluation of the results

The results were calculated and statistically analysed (Two-way ANOVA) using the GraphPad Prism software (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

9 STUDY RESULTS

9.1 Graphical presentation of DERP1 amount in house dust before and after application of BED Pro-biotic

The amount of the DERP1 allergen found in the analyzed dust samples was calculated and presented graphically as columns in Figure 2. The raw data from the absorbance measurement are presented in Table 2 and Appendix 2. In Table 1 are presented the data used for generation of Graph 1 – amount of DERP1 in U/ml calculated from the standard curve obtained with the standards in the DERP1 ELISA Kit. All data were processed and calculated as recommended by the manufacturer of the DERP1 ELISA Kit.



Graph 1. DERP1 allergen in dust samples collected in five different rooms every 5th day within a month.

Table 1. Amount of DERP1 [U/mL] calculated from the standard curve:

Room	Day 1		Day 5		Day 10		Day 15		Day 20	
R1	12.28264	11.69584	7.001467	8.585819	5.475795	5.221516	1.172616	1.857213	0.057702	0.135941
R2	12.3022	12.43912	7.236186	7.666504	4.947677	5.612714	3.148166	2.756968	0.233741	0.840098
R3	10.20929	13.20196	7.157946	8.116381	5.710513	5.534474	2.228851	2.541809	0.038142	0.018582
R4	12.53692	12.5956	7.079707	7.666504	5.632274	4.45868	2.639609	2.580929	0.898778	0.37066
R5	12.43912	13.22152	7.275306	8.116381	5.554034	4.4	2.600489	3.245966	0.703178	0.820538

Legend: R = room.



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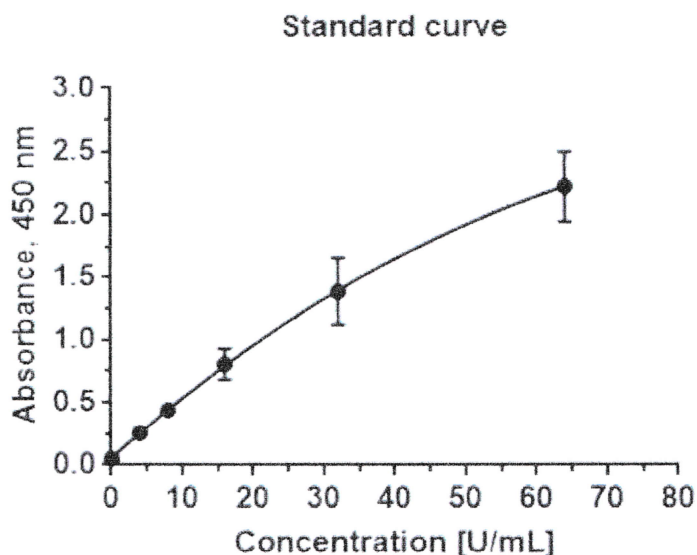
Table 2. Raw data from the from the absorbance measurement

Room	Day 1		Day 5		Day 10		Day 15		Day 20	
R1	0.68	0.65	0.41	0.491	0.332	0.319	0.112	0.147	0.055	0.059
R2	0.681	0.688	0.422	0.444	0.305	0.339	0.213	0.193	0.064	0.095
R3	0.574	0.727	0.418	0.467	0.344	0.335	0.166	0.182	0.054	0.053
R4	0.693	0.696	0.414	0.444	0.34	0.28	0.187	0.184	0.098	0.071
R5	0.688	0.728	0.424	0.467	0.336	0.277	0.185	0.218	0.088	0.094

Legend: R = room.

9.2 Standard curve for calculation of DERP1 amount [U/mL]



The standard curve was generated in GrapPad Prism with a XY table, which served for calculation of the amount of DERP1 in each sample. The standards for generation of the standard curve are included in the ELISA kit used for this study.



Graph 2. Standard curve used for calculation of the DERP1 amount in the dust samples.

Table 3. Raw data for the standard curve.

Concentration [U/mL]	Absorbance	
0	0.05	0.053
4	0.274	0.238
8	0.465	0.406
16	0.892	0.715

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32	1.575	1.193
64	2.029	2.414

9.3 Statistical analysis

The statistical analysis was performed with Two-way ANOVA.

Table 4. Comparison of the data regarding the time (columns) of sample collection for each room.



Within each row, compare columns (simple effects within rows)					
Number of families	5				
Number of comparisons per family	10				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
R1					
Day 5 vs. Day 1	-4.196	-6.023 to -2.368	Yes	****	<0.0001
Day 10 vs. Day 1	-6.641	-8.468 to -4.813	Yes	****	<0.0001
Day 15 vs. Day 1	-10.47	-12.3 to -8.647	Yes	****	<0.0001
Day 20 vs. Day 1	-11.89	-13.72 to -10.06	Yes	****	<0.0001
Day 10 vs. Day 5	-2.4454	-4.272 to -0.6175	Yes	**	0.0056
Day 15 vs. Day 5	-6.279	-8.106 to -4.451	Yes	****	<0.0001
Day 20 vs. Day 5	-7.697	-9.524 to -5.869	Yes	****	<0.0001
Day 15 vs. Day 10	-3.834	-5.661 to -2.006	Yes	****	<0.0001
Day 20 vs. Day 10	-5.252	-7.079 to -3.424	Yes	****	<0.0001
Day 20 vs. Day 15	-1.418	-3.246 to 0.4094	No	ns	0.1790
R2					
Day 5 vs. Day 1	-4.919	-6.747 to -3.092	Yes	****	<0.0001
Day 10 vs. Day 1	-7.09	-8.918 to -5.263	Yes	****	<0.0001
Day 15 vs. Day 1	-9.418	-11.25 to -7.591	Yes	****	<0.0001
Day 20 vs. Day 1	-11.83	-13.66 to -10.01	Yes	****	<0.0001
Day 10 vs. Day 5	-2.171	-3.999 to -0.3437	Yes	*	0.0151
Day 15 vs. Day 5	-4.499	-6.326 to -2.671	Yes	****	<0.0001
Day 20 vs. Day 5	-6.914	-8.742 to -5.087	Yes	****	<0.0001
Day 15 vs. Day 10	-2.328	-4.155 to -0.5002	Yes	**	0.0086
Day 20 vs. Day 10	-4.743	-6.571 to -2.916	Yes	****	<0.0001
Day 20 vs. Day 15	-2.416	-4.243 to -0.5882	Yes	**	0.0062
R3					
Day 5 vs. Day 1	-4.068	-5.896 to -2.241	Yes	****	<0.0001
Day 10 vs. Day 1	-6.083	-7.911 to -4.256	Yes	****	<0.0001
Day 15 vs. Day 1	-9.32	-11.15 to -7.493	Yes	****	<0.0001
Day 20 vs. Day 1	-11.68	-13.5 to -9.85	Yes	****	<0.0001
Day 10 vs. Day 5	-2.015	-3.842 to -0.1872	Yes	*	0.0263
Day 15 vs. Day 5	-5.252	-7.079 to -3.424	Yes	****	<0.0001
Day 20 vs. Day 5	-7.609	-9.436 to -5.781	Yes	****	<0.0001
Day 15 vs. Day 10	-3.237	-5.065 to -1.41	Yes	***	0.0003
Day 20 vs. Day 10	-5.594	-7.422 to -3.767	Yes	****	<0.0001
Day 20 vs. Day 15	-2.357	-4.184 to -0.5295	Yes	**	0.0077

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R4					
Day 5 vs. Day 1	-5.193	-7.021 to -3.366	Yes	****	<0.0001
Day 10 vs. Day 1	-7.521	-9.348 to -5.693	Yes	****	<0.0001
Day 15 vs. Day 1	-9.956	-11.78 to -8.129	Yes	****	<0.0001
Day 20 vs. Day 1	-11.93	-13.76 to -10.1	Yes	****	<0.0001
Day 10 vs. Day 5	-2.328	-4.155 to -0.5002	Yes	**	0.0086
Day 15 vs. Day 5	-4.763	-6.59 to -2.935	Yes	****	<0.0001
Day 20 vs. Day 5	-6.738	-8.566 to -4.911	Yes	****	<0.0001
Day 15 vs. Day 10	-2.435	-4.263 to -0.6077	Yes	**	0.0058
Day 20 vs. Day 10	-4.411	-6.238 to -2.583	Yes	****	<0.0001
Day 20 vs. Day 15	-1.976	-3.803 to -0.1481	Yes	*	0.0302
R5					
Day 5 vs. Day 1	-5.134	-6.962 to -3.307	Yes	****	<0.0001
Day 10 vs. Day 1	-7.853	-9.681 to -6.026	Yes	****	<0.0001
Day 15 vs. Day 1	-9.907	-11.73 to -8.08	Yes	****	<0.0001
Day 20 vs. Day 1	-12.07	-13.9 to -10.24	Yes	****	<0.0001
Day 10 vs. Day 5	-2.719	-4.546 to -0.8914	Yes	**	0.0020
Day 15 vs. Day 5	-4.773	-6.6 to -2.945	Yes	****	<0.0001
Day 20 vs. Day 5	-6.934	-8.761 to -5.107	Yes	****	<0.0001
Day 15 vs. Day 10	-2.054	-3.881 to -0.2263	Yes	*	0.0229
Day 20 vs. Day 10	-4.215	-6.043 to -2.388	Yes	****	<0.0001
Day 20 vs. Day 15	-2.161	-3.989 to -0.3339	Yes	*	0.0157

Table 5. Comparison of the data regarding the rooms (raws) of sample collection for each time point.

Within each column, compare rows (simple effects within columns)					
Number of families	5				
Number of comparisons per family	10				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Day 1					
R1 vs. R2	-0.3814	-2.223 to 1.46	No	ns	0.9724
R1 vs. R3	0.2836	-1.558 to 2.125	No	ns	0.9908
R1 vs. R4	-0.577	-2.418 to 1.264	No	ns	0.8863
R1 vs. R5	-0.8411	-2.682 to 1	No	ns	0.6688
R2 vs. R3	0.665	-1.176 to 2.506	No	ns	0.8244
R2 vs. R4	-0.1956	-2.037 to 1.646	No	ns	0.9978
R2 vs. R5	-0.4597	-2.301 to 1.382	No	ns	0.9466
R3 vs. R4	-0.8606	-2.702 to 0.9807	No	ns	0.6499
R3 vs. R5	-1.125	-2.966 to 0.7166	No	ns	0.3991
R4 vs. R5	-0.2641	-2.105 to 1.577	No	ns	0.9930
Day 5					
R1 vs. R2	0.3423	-1.499 to 2.184	No	ns	0.9814
R1 vs. R3	0.1565	-1.685 to 1.998	No	ns	0.9991

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R1 vs. R4	0.4205	-1.421 to 2.262	No	ns	0.9609
R1 vs. R5	0.0978	-1.744 to 1.939	No	ns	0.9999
R2 vs. R3	-0.1858	-2.027 to 1.655	No	ns	0.9982
R2 vs. R4	0.07824	-1.763 to 1.92	No	ns	>0.9999
R2 vs. R5	-0.2445	-2.086 to 1.597	No	ns	0.9948
R3 vs. R4	0.2641	-1.577 to 2.105	No	ns	0.9930
R3 vs. R5	-0.05868	-1.9 to 1.783	No	ns	>0.9999
R4 vs. R5	-0.3227	-2.164 to 1.519	No	ns	0.9851
Day 10					
R1 vs. R2	0.06846	-1.773 to 1.91	No	ns	>0.9999
R1 vs. R3	-0.2738	-2.115 to 1.567	No	ns	0.9920
R1 vs. R4	0.3032	-1.538 to 2.144	No	ns	0.9882
R1 vs. R5	0.3716	-1.47 to 2.213	No	ns	0.9749
R2 vs. R3	-0.3423	-2.184 to 1.499	No	ns	0.9814
R2 vs. R4	0.2347	-1.607 to 2.076	No	ns	0.9955
R2 vs. R5	0.3032	-1.538 to 2.144	No	ns	0.9882
R3 vs. R4	0.577	-1.264 to 2.418	No	ns	0.8864
R3 vs. R5	0.6455	-1.196 to 2.487	No	ns	0.8393
R4 vs. R5	0.06846	-1.773 to 1.91	No	ns	>0.9999
Day 15					
R1 vs. R2	-1.438	-3.279 to 0.4037	No	ns	0.1804
R1 vs. R3	-0.8704	-2.712 to 0.9709	No	ns	0.6404
R1 vs. R4	-1.095	-2.937 to 0.746	No	ns	0.4251
R1 vs. R5	-1.408	-3.25 to 0.433	No	ns	0.1960
R2 vs. R3	0.5672	-1.274 to 2.409	No	ns	0.8924
R2 vs. R4	0.3423	-1.499 to 2.184	No	ns	0.9814
R2 vs. R5	0.02934	-1.812 to 1.871	No	ns	>0.9999
R3 vs. R4	-0.2249	-2.066 to 1.616	No	ns	0.9962
R3 vs. R5	-0.5379	-2.379 to 1.303	No	ns	0.9094
R4 vs. R5	-0.313	-2.154 to 1.528	No	ns	0.9867
Day 20					
R1 vs. R2	-0.4401	-2.281 to 1.401	No	ns	0.9541
R1 vs. R3	0.06846	-1.773 to 1.91	No	ns	>0.9999
R1 vs. R4	-0.5379	-2.379 to 1.303	No	ns	0.9094
R1 vs. R5	-0.665	-2.506 to 1.176	No	ns	0.8244
R2 vs. R3	0.5086	-1.333 to 2.35	No	ns	0.9247
R2 vs. R4	-0.0978	-1.939 to 1.744	No	ns	0.9999
R2 vs. R5	-0.2249	-2.066 to 1.616	No	ns	0.9962
R3 vs. R4	-0.6064	-2.448 to 1.235	No	ns	0.8672
R3 vs. R5	-0.7335	-2.575 to 1.108	No	ns	0.7679
R4 vs. R5	-0.1271	-1.968 to 1.714	No	ns	0.9996

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10 CONCLUSION



The results obtained showed that BED Pro-biotic reduce the amount of DERP1 allergen in the samples significantly after the first application. Five days after the 4th application almost 100% reduction of the allergen in the samples was observed. The differences are significant between the days of sample collection. The results obtained were the same for each room (no significant difference results between the different rooms was calculated).

This result confers only to the tested three products and any extrapolation or transfer to other products is responsibility of Perilis Trading EOOD.

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12 APPENDICES

12.1 Appendix 1: laboratory equipment, consumables, media and reagents.

Laboratory equipment

Device	Manufacturer	Model	Serial number	Date of last prophylaxis/calibration
Incubator	Panasonic, Japan	MCO-18AC-PE	14080350	04.02.2020/Operating parameters protocol/ELTA'90
Centrifuge	Hermle, Germany	Z 206 A	601	04.02.2020/Protocol of findings RD10.4/ELTA'90
ELISA-Reader	BioTek	Elx800	141208C	04.02.2020/Protocol of findings RD10.4/ELTA'90
Data-logger	ETI, UK	ThermaData TD2F	296-111	Certificate Nr. 50-LK/30.01.2020

Disposable medical consumables



Material	Description/Manufacturer	Cat. Nr.	Lot. Nr.	Expiry date
Pipette tips	Gamma-sterilized, 200 µl, 96 per box	T-200-Y-R-S	17719402	26.06.2022
Pipette tips	Gamma-sterilized, 1000 µl, 96 per box	T-1000-B-R-S	22319425	11.08.2022
Serological pipettes	Gamma-sterilized, plastic, 5 ml, individually wrapped, Corning	4487	16518051	13.06.2021
Combi advanced 0.5 ml	Eppendorf	0030089634	H1795320	09.2023
Combi advanced 5 ml	Eppendorf	2433	H1793600	09.2023
Duafilter 5 ml L	Eppendorf	0030077725	F168409N - 1430	-

Pipettes

Material	Description	Model/Manufacturer	Calibration date
Pipette	Variable, single-channel, 500-5000 µl	Eppendorf Research Plus P32486D	03.04.2019
Pipette	Variable, single-channel, 100-1000 µl	Biohit Proline 14647911	03.04.2019
Pipette	Variable, single-channel, 20-200 µl	Axygen AP-200 358150004	03.04.2019
Pipette	Variable, single-channel, 0.5-10 µl	Axygen AP-10 458120157	03.04.2019
Dispenser	for combitips from 0.1 to 24 ml	Eppendorf Multipette M4 P42999D	03.04.2019
Stripettor	For serological pipettes, filter 0.45 µm	Corning, Stripettor 4099	-

Media, buffers, solutions and reagents

Material	Description/Manufacturer	Cat. Nr.	Lot. Nr.	Expiry date
DPBS	Gibco	14190-086	2007043	30.06.2021
DERP1 ELISA kit	BioSource, Inc.	#MBS771220	#201906	12.2019

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12.2 Appendix 2: Absorbance – raw data

Separate file in Excel format.

Date of preparation: 20.05.2020

Prepared by:.....

/Assist. Prof. Maya M. Zaharieva,
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Date of approval: 22.05.2020

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Date:.....05.06.2020

