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I TITLE PAGE

Test Facility	Assignor
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1113 Sofia, Bulgaria	Municipality Aksakovo, Village Kichevo
	Agricultural cooperative ZORA

Antimicrobial activity of probiotic products:

1. Test of the parallel streaks

2. Agar diffusion method

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3 SUMMARY

Three probiotic products of Perilis Trading EOOD were investigated for antimicrobial activity on five pathogenic bacterial strains and one pathogenic fungal strain using two different approaches for evaluation of the antimicrobial susceptibility of the pathogenic strains. On one hand, the method of the parallel streaks was used to determine the potential of the probiotic products to inhibit the growth of the pathogenic strains in conditions of concurrence in the same nutrition milieu. On the other hand, the agar diffusion test was applied in order to determine the potential of filtrates obtained from the products to inhibit the growth of the selected pathogen which would be indicative for the production of antimicrobial molecules.

The results obtained showed that all three products inhibited competitively the growth of the Gram-positive pathogenic bacterial strains and the fungus *Candida albicans* plated in parallel streaks near the band of the probiotic microoroganisms. The filtrate obtained from product 2 (odor neutralizer) inhibited to a high extend (>24 mm inhibition zone) the growth of Gram-positive strains *Streptococcus pyogenes*, *Enterococcus faecalis* and *Staphylococcus aureus* and slightly the growth of C. albicans (10 mmm inhibition zone). The products did not show any effect on Gram-negative bacterial strains.

4 LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

Test sample: product to be tested for antimicrobial activity

BHI: brain heart infusion

BHIA: brain heart infusion agar

MHA: Mueller Hinton Agar

5 INVESTIGATORS AND STUDY ADMINISTRATIVE STRUCTURE

5.1 Inverstigators

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Assist. Prof. Maya M. Zaharieva, PhD

Microbiologist Iva Tsvetkova

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5.2 Administrative structure of the study

Figure 1. Administrative structure of the study

6 INTRODUCTION

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 [1]. 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, 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 [2]. Bacilli are stable during processing and storage of food, pharmaceutical and other preparations, which makes 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 [3].

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The current study is focused on the evaluation of the antimicrobial activity of three probiotic products based on *Bacillus* strains and intended for use in the human life and household. Our hypothesis for the antimicrobial potential of these products 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 antimicrobial activity of the following three probiotic products of the company Perilis Trading EOOD:

1) BED Pro-biotic, van der Schoot technology – probiotic neutralizer for mites and allergens
2) ROOM Pro-biotic, van der Schoot technology – hypoallergenic odour neutralizer
3) SHOES Pro-biotic, van der Schoot technology – probiotic odour neutralizer for shoes

As test microorganisms for the tasks execution were selected the following standard pathogenic bacterial strains from the American Type Culture Collection (ATCC), recommended in the ISO 20776-1/2006 for testing of antimicrobial activity:

1) *Staphylococcus aureus,* ATCC 29213,
2) *Enterococcus faecalis,* ATCC 29212,
3) *Escherichia coli,* ATCC 35218,
4) *Pseudomonas aeruginosa,* ATCC 27853,

and the following two strains from the collection of the Stephan Angeloff Institute of Microbiology (SAIM) at the Bulgarian Academy of Sciences:

1) *Streptococcus pyogenes*, SAIM 10535,
2) *Candida albicans*, CBS 562, The Netherlands.

7.2 Evaluated parameters

- **Zone of inhibition**

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8 INVESTIGATIONAL PLAN

8.1 Description of the test samples

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

Name of product 1: BED Pro-biotic, van der Schoot technology – probiotic neutralizer for mites and allergens

Name of product 2: ROOM Pro-biotic, van der Schoot technology – hypoallergenic odour neutralizer

Name of product 3: SHOES Pro-biotic, van der Schoot technology – probiotic odour neutralizer for shoes

Identification number of product 1: LOT...

Identification number of product 2: LOT...

Identification number of product 3: LOT...

Expiry date of product 1: 12.2020

Expiry date of product 2: 12.2020

Expiry date of product 3: 12.2020

Content of product 1: Fermentative bacteria < 5 %, hypoallergenic perfume composition

Content of product 2: Fermentative bacteria < 5 %, hypoallergenic perfume composition

Content of product 3: Fermentative bacteria < 5 %, hypoallergenic perfume composition

Intended use of product 1: 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.

Intended use of product 2: removes successfully unpleasant odours such as cigarette smoke, fat, urine, sweat, perfumes, and pets. It inhibits the growth of pathogenic bacteria and contributes to a healthy home microclimate.

Intended use of product 3: removes successfully unpleasant odours of shoes and significantly decreases the risk for development of fungal infections of the feet.

Package of product 1: A white, non-transparent 500 ml plastic spray bottle.

Package of product 2: A white, non-transparent 500 ml plastic spray bottle.

Package of product 3: A white, non-transparent 100 ml plastic spray bottle.

Conditions for storage of products 1, 2 and 3: At temperature between 5 and 35 °C.

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8.2 Study design and methods

Two tests were performed to evaluate the antimicrobial activity of the test samples:

1) Test of the parallel streaks and
2) Agar diffusion assay

The study design is presented in the following diagram:

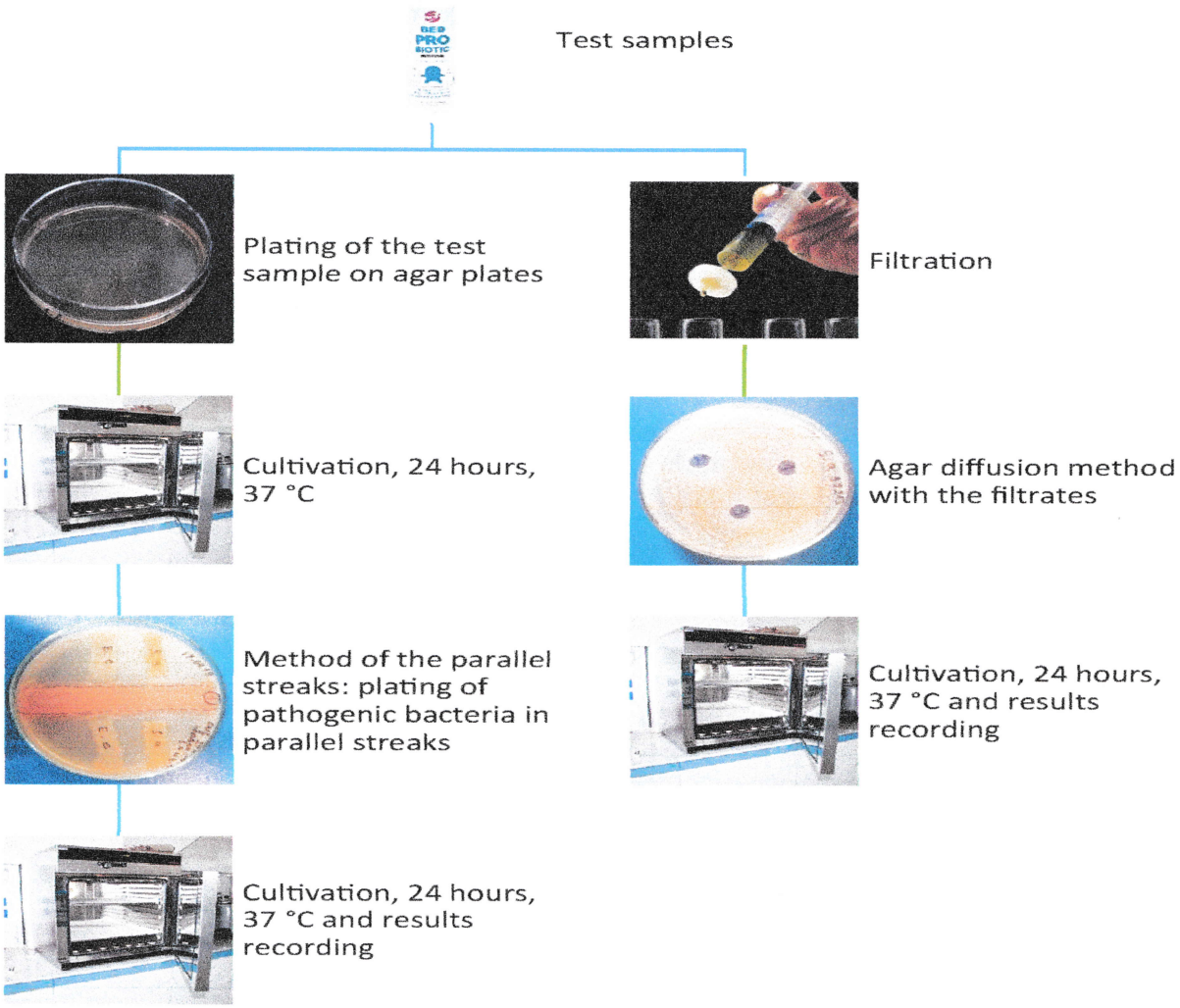

Figure 2. Study design and methods

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8.3 Study performance

8.3.1 Preparation of the agar media

Both assays were performed on BHIA or MHA for the bacterial strains and Sabouraud agar for *Candida albicans*.

All media were be prepared from commercially available dehydrated bases according to the manufacturer's instructions. Immediately after autoclaving, the medium was allowed to cool in a 45 to 50 °C water bath. The freshly prepared and cooled medium was poured into glass flat-bottomed petri dishes (100 mm) on a level, horizontal surface to give a uniform depth of approximately 4 mm, which corresponds to 25 ml for a plate. The agar medium was cooled to room temperature and, unless the plates were used the same day, stored in a refrigerator (2 to 8 °C). Plates were used within seven days after preparation. A representative sample of each batch of plates was examined for sterility by incubating at 30 or 37 °C for 24 hours.

8.3.2 Preparation of the test samples

For the method of the parallel streaks, the test samples containing probiotic microorganisms were plated each of them on a agar plate along the diameter of the plate with a width of 0.5-1 cm. Thereafter, the plates were cultivated for 24 hours at 37 °C to allow the bacteria to grow into a dens band before testing their antimicrobial activity.

For the agar diffusion test, the test samples were sterile filtered through a sterile filter 0.2 µm under sterile conditions (Laminar Air Flow Telstar Bio II Advance, Spain) and 100 µl of each filtrate were used for evaluation of antimicrobial activity.

8.3.3 Inoculation of the pathogenic test bacteria

At least three to five isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml of a suitable broth medium. The broth culture was incubated at 37 °C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually not more than 18 hours). The turbidity of the actively growing broth culture was adjusted with sterile saline to obtain a turbidity optically comparable to that of the 0.5 McFarland standard which corresponds to a suspension containing approximately 1 to 2 x 10^8 CFU/ml. The inoculum tube was compared visually to a tube containing 0.5 McFarland standard against a card with a white background and contrasting black lines. The inoculum suspension was used within 15 minutes after adjusting the turbidity.

For the method of the parallel streaks, this suspension was plated directly on the agar plate in streaks parallel to the already grown probiotic microorganisms and near to its edge.

For the agar diffusion tests, a sterile cotton swab was dipped into the adjusted suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of the agar plate was inoculated by streaking

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the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow any excess surface moisture to be absorbed before cutting the reservoirs and applying the filtrates.

8.3.4 Incubation with the test samples

For the method of the parallel streaks, the plates inoculated with the the pathogenic test strains (p. 8.3.3) were incubated for 24 hours at 37 °C before reading the result.

For the agar diffusion test, after the inoculation 7 mm diameter holes were cut in the agar gel and filled with 100 µl of the tested filtrate. Thereafter, the plates were left for 2 hour at 4 °C to allow the inoculum to penetrate the agar and the plates were transferred to an incubator and cultured for 24 hours at 37 °C before reading the result.

8.3.5 Recording of the results

The results were read at the 24th hour of the incubation period, whereby the zones of inhibition were measured in mm. The results were photo-documented and the pictures are presented in figures in section Results.

8.4 Evaluation of the results

The inhibition zones were measured in mm. The bigger the inhibition zone, the more susceptible was the test pathogenic microorganism to the antagonistic effect of the probiotic strains in the respective test sample.

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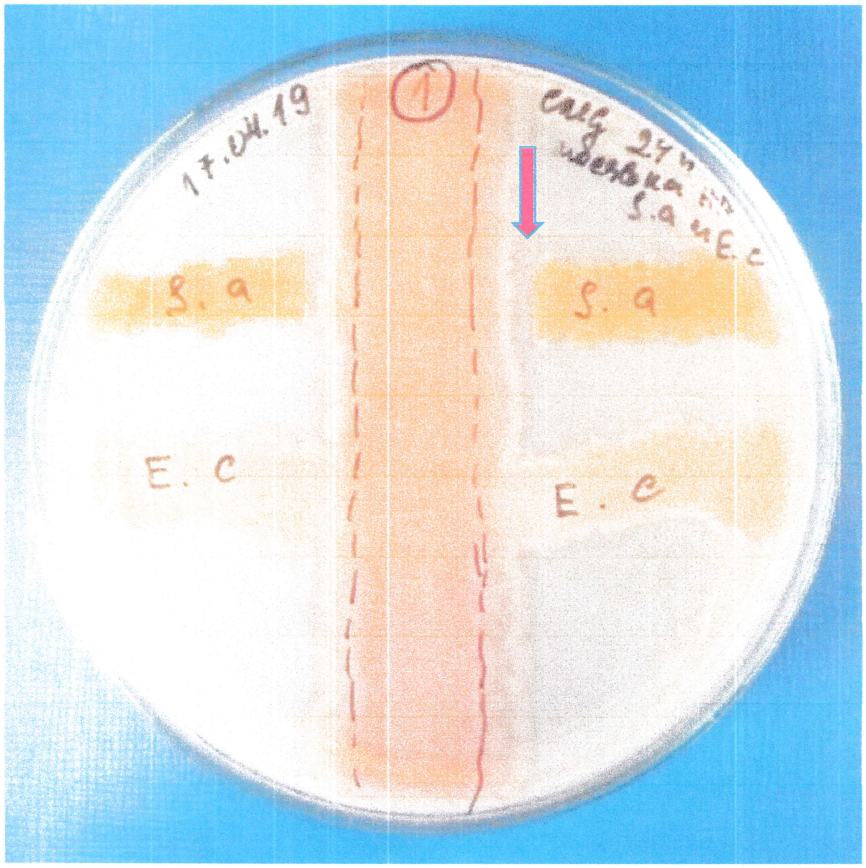
9 STUDY RESULTS

The results obtained from both tests are presented in figures as follows:

9.1 Results from the parallel streak assay

Figure 3A. Product 1 – inhibition of *Staphylococcus aureus* and *Escherichia coli* evaluated through the method of the parallel streaks.



As visible from Figure 3A there is an inhibition zone between the probiotic strain and the Gram-positive strain *S. aureus* which indicates for the inhibitory potential of the probiotic strains in product 1 against pathogenic staphylococci.