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Bacterial Agents for Biocontrol of American Foulbrood (AFB) of Larvae Honey Bee

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Abstract: Bee colonies that belong to the biocenosis are constantly threatened by the bacterial larvae disease called American foulbrood, caused by *Paenibacillus larvae*. The current state of the law prevents the inclusion of pharmacotherapy in the treatment and the only control is based on physical eradication of infected colonies by order of the Vet. The purpose of this study was to investigate the antimicrobial activity of selected bacteria with proven probiotic potential that could reduce American foulbrood pressure by promoting the development of the larvae microbiome that competes with and blocks the excessive proliferation and production of *P. larvae* endospores. The antimicrobial activity of inoculants of selected strains was studied: B. *pumilus, B. licheniformis, S. narbonensis, L. fusiformis, L. brevis, B. megaterium* against *P. larvae* ATCC 9545 and *P. larvae* CCUG 48973. Analyses were performed by the well-diffusion method according to EUCAST standards with modifications due to the specificity of the bacteria used. The largest zone of growth inhibition of *P. larvae* was confirmed with: *S. narbonensis, B. licheniformis, B. megaterium*, medium activity was observed with *L. brevis* and *B. pumilus*. Negligible activity was shown by *L. fusiformis*. Differences were noticed in the resistance of indicator strains of *P. larvae* and between the media and carriers used.

Keywords: AFB; Paenibacillus; larvae; bees; probiotics; biocontrol; biosecurity; beneficial; microorganisms; bioasecuration

1. Introduction

The work of honeybee workers for humans and the ecosystem is invaluable. EFSA reports that the annual pollination by bees has been valued at around 22 billion Euros, and in Poland alone, this value exceeds 964 million Euros [1,2]. Worldwide, the economic value of pollination with honeybee workers has been estimated at 265 billion Euros per year [3,4]. Other sources report that the global value of pollination involving honeybee workers is estimated to be between US$ 235-577 billion and up to half a trillion dollars [5–8]. Researchers have been searching for many years for factors that reduce losses in apiary management and increase the security of the honeybee as the main pollinator in the food production chain [9–11]. Bee colonies are constantly threatened by brood and adult bee diseases, susceptibility to xenobiotics (including pesticides), an impoverished forage base, lack of diverse protein food resulting in massive worker malnutrition (nutritional stress), environmental stress, and newer and newer pests arriving from different parts of the world (populations of the small hive beetle or Asian hornet) [12,13]. The honey bee is also accompanied by a microflora whose biodiversity is modified by many environmental factors, including stress factors such as pesticides, forage shortages, pressure from the pathogenic microbiome, and immune impairment [14–16]. According to various literature sources, there are between 6,000 and 8,000 different microbial strains in the microbiome of bee colonies [17].

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One microbial threat to the balance of the colony microbiome is *Paenibacillus larvae*—a Gram-positive, peridomestic, mobile, virulent, spore-forming larvae that cause a brood disease called American foulbrood [18]. The bacteria *Paenibacillus larvae*, which produces approximately one billion spores per infected larva, is the main vector of this disease [19]. Only spores can cause infection. Spores can survive for up to 50 years under favorable conditions and exhibit infectious properties for 35 years. A temperature of 100°C destroys spores after 5 days, while at 140-170°C they die after only 2 hours. Spores are also killed in 5-10% formalin within 6 hours, while soda lye kills spores at a concentration of 2% within 4 minutes. 5% sodium hypochlorite is also effective. Vegetative forms die at 60°C [20–22]. An infected and disease-dead larva can contain up to 2.5 billion endospores, but only 10 to 35 spores are needed to infect another larva, indicating high virulence and a high rate of disease spread [23,24]. Spores can reside in honey, wax, royal jelly, propolis, pollen, and bee feathers. Once in the digestive tract of the larvae, they germinate after 24 hours, leading to bacterial superinfection manifested by rapid growth of vegetative forms. The vegetative cells damage the epithelium and intestinal walls and later attack all internal organs leading to cytolysis and histolysis [25–27]. Nine days after infection, spores begin to form. The maggots die after 2-3 days after the cell is sealed [28]. The dead larvae become flaccid, changing color first to yellow, then to a brown, sticky, and malleable mass. Five serotypes of American foulbrood are currently recognized worldwide: Eric I, Eric II, Eric III, Eric IV, and Eric V [29,30].

The epizootiological agent for all varieties remains the same bacterial species: *Paenibacillus larvae*. Sick bee colonies are physically eradicated on the recommendation of veterinarians, legislation in the EU does not allow the use of any form of pharmacotherapy, and in Poland, American foulbrood is eradicated ex officio. In the past, antibiotics and sulphonamides were used. Currently, only administrative procedures are available for American foulbrood control [31]. Once American foulbrood is confirmed, the district veterinarian may decide to eradicate the colonies or, in justified cases, order a double relocation procedure to a new or decontaminated hive on frames with hoses [32]. Most cases with confirmed cases of foulbrood are classified as a high-risk group and end up with the burning of infected colonies. Prevention consists primarily of maintaining appropriate sanitary standards involving, for example, frequent changes of combs, and disinfection of hives and tools (e.g., with Virkon S). Other prevention methods include descriptions of the use of essential oils: cinnamon, rosemary, thyme, lemon or aniseed. The effect of extracts of various herbal species on *P. larvae* cells is also known from in vitro laboratory studies [33,34]. Solutions described as effective in reducing *P. larvae* abundance include products to be applied as food additives (syrup or cake) and products to be applied as sprays or sprinkled on the inter-frame streets [35].

The Institute of Veterinary Medicine in Poland reports that *P. larvae* spores are present in between 23 and 45% of the cases analyzed. Infected colonies weaken very quickly, the number of spring decreases dramatically leading to so-called colony unsealing, the number of bees decreases, which leads to a reduction in the economy of the farm or the total liquidation of the apiary. Statistical data from the Veterinary Inspectorate in Poland indicates several hundred cases of detected outbreaks, but these data are unreliable and underestimated and differ from the actual state. In previous years, i.e., 2009-2011, screening for the occurrence of *Paenibacillus larvae* in bee colonies was carried out in 162 districts belonging to 9 voivodships. 6 510 honey samples were taken from 32 550 bee colonies from 2 294 apiaries. The disease was diagnosed in 45% of all apiaries examined, and the greatest intensity occurred in the Małopolskie voivodeship. In the Warmińsko-Mazurskie voivodeship, the presence of the bacteria was confirmed in 58% of the apiaries examined. Between 26% and 47% of apiaries were contaminated with *P. larvae* spores [36]. An even earlier history of the occurrence of American foulbrood in Poland is described in research studies conducted in 2005 and 2007. In 2005, out of 142 samples, 34 tested positive for *P. larvae*, meaning that the bacteria were present in 23% of all samples. Similar statistics were found in 2007, where 23 out of 100 tests were positive. The number of Aedospores in 1 gram of honey ranged from 10 to more than 1,000. Such a huge scale of infections, still increasing from year to year, shows that the Polish beekeeping environment requires and needs the immediate implementation of a solution to reduce the uncontrolled growing number of infections in apiaries by supporting the bees' natural protective

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barriers and stimulating increased nest hygiene [37]. The data obtained from the above-mentioned monitoring indicates a growing problem with *P. larvae* in bee colonies, which promotes the increasing incidence of American foulbrood. A high microbial burden on bee colonies is also observed in other countries, with a high number of infections also recorded in Austria [38].

Currently, the number of bee colonies in Poland is 2.35 million, so this is a threefold increase since 2005 (827.4 thousand bee families). Bee families are looked after by around 97,000 beekeepers. The average bee colony per km^2 is 7.50. Unfortunately, the ever-increasing degree of apiculture in Poland favors the circulation of diseases between bee colonies [39]. As of today, there are only 61 Veterinarians in Poland with a specialization in diseases of commercial insects [40]. In Poland, there are no specific legal restrictions on the movement of migratory apiaries, which exacerbates the epidemiological situation.

The phenomenon of competition between microorganisms is well-known. Bacterial metabolites, also known as metabiotics, have become an object of scientific interest in recent years. Micro-organisms isolated from the digestive tracts of bees with in vitro antimicrobial activity against *P. larvae* have been described in the literature [41,42]. Such activity may be called bioassurance. The definition given by Zygmunt Pejsak includes specific indications to be implemented to reduce or eliminate the risk of pathogen transmission into the flock. The aim of bioassurance is, among other things, to reduce the risk of introducing an infectious agent into the herd [43]. In the definition of another researcher Thomas Gillespie, bioassurance must reduce the risk of pathogen intrusion and must consist of bioexclusion, which will control the transmission of pathogens. This element of bioassurance has been referred to as biocontainment [44]. Bioassurance is the so-called biological protection consisting of protecting the animals through preventive and sanitary measures using biocontainment on the farm as well as in the immediate surroundings [45,46]. Adherence to bio-assurance aims, among other things, to reduce the incidence of pathogens and to improve animal health. Bioassurance is a set of measures aimed at maintaining or improving the health status of a herd through the use of specific organizational methods [47,48]. In other words, it is the biological protection of the farm. Effective bioassurance gives protection to the herd against the transmission of infectious agents [49–52]. Also, the Cambridge English Corpus states that these are the methods that are used to stop a disease or infection from spreading from one person, animal, or place to others or actions that are taken to prevent damage from biological threats using biological methods [53]. Biotisation as a tool for the introduction of bioassurance may be a new biotechnological approach to animal husbandry. It consists of inoculating the animal environment with beneficial micro-organisms such as fungi or bacteria to increase their dominance in the environmental microbiome by increasing the tolerance of animals to biotic and abiotic stresses (in practice this is spraying or fogging). Biotisation is the process of inoculating with symbiotic native bacteria (bacterization) or involves the introduction of beneficial fungi-yeasts (microzation) [54,55]. Beneficial micro-organisms also known as beneficial or effective microorganisms (EM) have an important function in restoring the microbial balance in the native microbiome of animals and also supply animals with valuable metabolites and protect them from pathogens. Biosanitisation is a process that seeks to eliminate as many pathogenic microorganisms as possible, most commonly on flat surfaces and in the animal environment. Sanitization is carried out using common cleaning agents or biopreparations. This process does not guarantee the sterility of the cleaned object but allows the degraded and sterilized animal microbiome to be inoculated and rebuilt [56,57]. In the case of bee colonies, this can include inoculation of nests and hives with probiotics as a permanent integral part of good beekeeping practice. Bacteriophage therapies appear to be some kind of innovation in reducing *P. larvae* infections, but phages are not officially authorized for use in Europe [58].

The lack of effective product solutions on the market to prevent the development of superinfection, the unstable administrative and legal status of American foulbrood, the risky practices of beekeepers and growers exacerbating the American foulbrood problem, the increased interest on the market in natural products like targeted microbial biopreparations with increased metabolite content, the untapped potential of biotechnology and microbiology in the control and

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prevention of livestock diseases and animal welfare are just some elements of why this topic of work was undertaken.

2. Materials and Methods

2.1. Research Problem

The complexity of the research problem concerns: (1) the reduction of American foulbrood pressure in bee colonies by supporting the development of the physiological microbiome of honeybee larvae, competing with and blocking the excessive proliferation and production of *P. larvae* endospores, and (2) testing the sensitivity of *P. larvae* vegetative cells to biologically active substances, constituted by biotechnological processes aimed at obtaining a high content of bioactive compounds in the final product using bacterial probiotic components.

2.2. Purpose of the Study

The aim of the study was: (1) to test the biological activity of selected bacterial components with confirmed probiotic potential that could constitute the composition of a pilot pre-formulation of a future metabolic preparation, and (2) to compare the antimicrobial activity of inoculants and post-culture (post-fermentation) fluids of selected, functional bacterial strains against *Paenibacillus larvae* ser. Eric I and Eric II.

2.3. Scope of Work

The overall scope of the work involves making inoculants of functional bacterial strains by revitalizing the bacterial strains by hydration of lyophilizes according to in-house procedures, then carrying out the actual multiplication (fermentation) in glass bioreactors and obtaining the first half pre-formulations on a laboratory scale. This consisted of transferring the functional bacteria cultures to selected media and multiplying by a technological process aimed at obtaining bioactive metabolites. The final step was to determine the antimicrobial activity of the culture fluids by determining the inhibition zones of the indicator strain. This was done by inoculating bloody agar with a suspension obtained from the selective multiplication of biological material on selective nutrient slants for *P. larvae*. Results were presented after 48-72 hours of incubation at 37°C.

2.4. Bacterial Isolates

2.4.1. Work Material

The working material consisted of the functional, bacterial strains which were used in an experiment to check their antimicrobial activity: *Bacillus pumilus* R5, *Bacillus licheniformis* M/542/M/18, *Streptomyces narbonensis* R71, *Lysinibacillus fusiformis* E23, *Levilactobacillus brevis* M/495/M/17, *Bacillus megaterium* R7. The strains were deposited at the Institute of Microbiological Technology in Turek, Poland, and subjected to deep-freezing at -80°C in glycerol. Isolation of the strains and species identification using 16S gene sequencing were carried out between 2014 and 2019. The morphology, physiology, and biochemical characteristics of the strains were very well described before the bacteria were banked using confocal microscopy, an API strip kit (BioMerieux), and the Biolog system (BioMaxima). The strains were from the wild environment from Poland and did not show virulence genes. The test strains had confirmed probiotic potential.

2.4.2. Biological Material

The biological material consisted of the bacterial, pathogenic, indicator strains belonging to *Paenibacillus larvae* ATCC 9545 (Labiol) and *Paenibacillus larvae* CCUG 48973 (Labiol). The strains were deposited at the Institute of Microbiological Technology in Turek, Poland, and subjected to deep-freezing at -80°C in glycerol. The strains were purchased for the experiment from an external supplier (Labiol) recommended by the district veterinary inspections. The supplier provided a protocol confirming the identification of the strains and a quality certificate with instructions for reviving the

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strains and the composition of the media. After revival, the species of the strains were control-confirmed by MALDI TOF/MS.

2.4.3. Cultivation of Microbial Cultures—Bacterial Inocula

The research and development work was divided into 2 stages. In stage 1, banked bacterial strains were revived. Colonies were fixed on appropriate media. Strains belonging to *Bacillus pumilus* R5, *Bacillus licheniformis* M/542/M/18, *Lysinibacillus fusiformis* E23, and *Bacillus megaterium* R7 were seeded on nutrient agar (Argenta) and incubation took place at 37°C under aerobic conditions. A strain belonging to *Streptomyces narbonensis* R71 was seeded on Streptomyces medium (Sigma-Aldrich) and incubation took place at 28°C under aerobic conditions. The strain belonging to *Levilactobacillus brevis* M/495/M/17 was seeded on MRS agar (Argenta) and incubation took place at 30°C under anaerobic conditions. All incubations were conducted for 24-48 hours (incubator BE400, Memmert, ZALMED, Poland). On reading the cultures, colony samples were control directed to MALDI-TOF/MS analyses to confirm species affiliation. Functional bacterial colonies were then transplanted onto agar-nutrient slants to derive young 24-hour colonies, incubated at specific temperature ranges for 24 hours under conditions appropriate to each strain (as above). At the end of the incubation, so-called microbial washes were made from the 24-hour cultures of pure bacterial cultures and inocula with an optical density of OD = 0.5° McFarland (approximately 1.0×10^8 CFU/cm^3 (densitometer DEN-1B, Biosan, Poland) were obtained.

2.4.4. Preparation of Microorganism Cultures in Bioreactors

The prepared inoculants were used to inoculate in the bioreactors (Donserv, LPP Equipment) an industrial medium (autoclaved 4% aqueous solution of organic cane molasses on distilled water) at 4% by volume and a second, liquid tryptone-pepton with glucose and yeast extract medium (BTL) also at 4%. Cultures were conducted in 18 litre glass bioreactors at 30-38°C for 10 days under anaerobic conditions for *L. brevis* and for other bacteria under aerobic conditions all the time monitoring biotechnological parameters such as analysis of kinetics of pH value changes, ORP-Redox conductivity [µs/cm], sugar content [°Brix], asepsis, general contamination (culture in buffered peptone water), aerobic microorganisms (culture on PCA medium before and after application of buffered peptone water, Argenta), fungi (DRBC before and after buffered peptone water, Argenta), lactic fermentation bacteria (MRS and MRS with 2% fructose before and after buffered peptone water, Argenta), *Clostridium sp*. (culture on ISA medium before and after treatment with buffered peptone water, Argenta). The culture process was considered complete with a marked decrease in pH, an increase in conductivity (ORP-Redox), a decrease in Brix, and an increase in culture density [CFU/cm^3].

2.5. Working Methodology

2.5.1. Agar Diffusion Assay

The second stage focused exclusively on screening and determination of antimicrobial activity against *P. larvae*. For the determination of antimicrobial activity, the well-diffusion method was used according to EUCAST standards with modifications due to the specificity of the bacteria used [59–61]. For this purpose, the *P. larvae* Eric I and Eric II strains were revived by transplanting colonies onto blood agar medium (Argenta) and then onto agar slants, respectively, incubated at 37°C for 24-48 h in a CO_2 atmosphere (incubator BE400, Memmert, ZALMED, Poland). A so-called microbial wash was then performed with an optical density of OD = 0.5° McFarland (approximately 1.0×10^8 CFU/cm^3, densitometer DEN-1B, Biosan, Poland). Each of the functional inoculants was combined respectively with the media used in beekeeping to feed the bee colonies (sterile water and sterile sucrose solutions of 50% and 60%, Merck), and mixtures of 3, 4, 5, 10% v/v were obtained. The culture fluid in undiluted form was treated to a concentration of 100%. Media without bacteria were used as control groups, i.e., sterile water and sterile sucrose solutions of 50% and 60% (Merck). Subsequently, surface cultures (1 ml) of *P. larvae* were inoculated using a microbial spatula on plates with blood

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agar and cut wells in the medium. 20 µl of the appropriate inoculant with carrier was transferred to the wells, and media not inoculated with inoculants was transferred to the control plates. All Petri dishes (Thermo Scientific) were incubated for 48-72 hours at 37°C in a CO_2 atmosphere (incubator BE400, Memmert, ZALMED, Poland). After the time had elapsed, readings were taken, and 'halo' zones were measured [62–65].

2.5.2. Antimicrobial Activity of Bacterial Suspensions against P. larvae

The tests were carried out in 3 replicate series considering each functional strain (6 strains) and a mixture of strains (1), different media (2 media), percentage concentration of culture fluid (5 concentrations), media of appropriate concentration (3 media), and each replicate series consisted of a total of 210 Petri dishes (Thermo Scientific) for all strains and mixtures tested.

2.6. Statistical Analysis

Statistical analysis of the results obtained was performed using one-way ANOVA (Tukey's parametric post-hoc test) at a significance level of p-value < 0.05. This analysis allows the means of any number of groups to be compared with each other, showing statistically significant similarities or statistically significant differences. The univariate scheme (one-way analysis of variance) tested whether one independent variable (factor) influenced the results of one dependent variable. The statistical analysis included results from the evaluation of antimicrobial potential (v/v concentrations of the inoculant—4 groups versus the carriers: water, sucrose 50%, and sucrose 60%—3 groups). In the scientific literature, results are also analyzed using Student's t-test, Mann-Whitney U-test, Katz's approximation, Pearson's Chi-square test, or Fisher's exact test [66–69].

3. Results

3.1. Preparation of Microorganism Cultures in Bioreactors

Strains belonging to the species: B. pumilus, B. licheniformis, S. narbonensis, L. fusiformis, L. brevis, and B. megaterium were successfully multiplied in a volume of 18 liters on target media. The culture lasted for 10 days. The release parameters met the final specifications of the process:

- *Bacillus pumilus* R5

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Strain to belong to the species *Bacillus pumilus* successfully multiplied in a volume of 18 liters on target media. The culture lasted for 10 days. The final culture density was 4.5 x 10^5 CFU/cm^3 in cane molasses medium to 4.7 x 10^5 CFU/cm^3 in tryptone-peptone medium. There is a marked decrease in pH values, a slight decrease in ORP-Redox, and a slight consumption of sugars manifested by a weak decrease in Brix values. No infections with foreign microflora are observed [Table 1].

Table 1. Reactor culture parameters for *Bacillus pumilus R5* using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Bacillus pumilus* R5											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,31	7,10	5,22	7,30
	Conductivity [µs/cm]	·	·	·	·	·	·	2800	2788	2810	2600
	Brix	·	·	·	·	·	·	3,7	3,7	3,6	3,4
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	4,87	6,50	4,67	6,70
	Conductivity [µs/cm]	·	·	·	·	·	·	2607	2500	2540	2700
	Brix	·	·	·	·	·	·	3,6	3,4	3,5	3,5
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	$3,3x10^5$ CFU/cm^3	$3,5x10^5$ CFU/cm^3
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	$4,5x10^5$ CFU/cm^3	$4,7x10^5$ CFU/cm^3
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3

- *Bacillus licheniformis* M/542/M/18

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Strain belonging to the species *Bacillus licheniformis* successfully multiplied in a volume of 18 liters on target media. The culture lasted for 11 days. The final culture density was 3.5 x 10^7 CFU/cm^3 in cane molasses medium to 3.7 x 10^7 CFU/cm^3 in tryptone-peptone medium. There is a marked decrease in pH values, a significant increase in ORP-Redox, and little consumption of sugars manifested by a weak decrease in Brix values. No infections with foreign microflora are observed [Table 2].

Table 2. Reactor culture parameters for *Bacillus licheniformis* M/542/M/18 using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Bacillus licheniformis* M/542/M/18											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,00	7,12	5,18	7,20
	Conductivity [µs/cm]	·	·	·	·	·	·	2200	2400	2220	2410
	Brix	·	·	·	·	·	·	3,7	3,7	3,8	3,8
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	4,37	6,40	4,47	6,60
	Conductivity [µs/cm]	·	·	·	·	·	·	3030	2900	2940	2800
	Brix	·	·	·	·	·	·	3,6	3,5	3,7	3,5
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	3,1x10^7 CFU/cm^3	3,6x10^7 CFU/cm^3
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	3,5x10^7 CFU/cm^3	3,7x10^7 CFU/cm^3
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3

- *Streptomyces narbonensis* R71

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Strain belonging to the species *Streptomyces narbonensis* successfully multiplied in a volume of 18 liters on target media. The culture lasted for 12 days. The final culture density was 4.5 x 10^6 CFU/cm^3 in reed molasses medium to 4.7 x 10^6 CFU/cm^3 in tryptone-peptone medium. There is a marked decrease in pH values, a significant increase in ORP-Redox, and little consumption of sugars manifested by a weak decrease in Brix values. No infections with foreign microflora are observed [Table 3].

Table 3. Reactor culture parameters for *Streptomyces narbonensis* R71 using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Streptomyces narbonensis* R71											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,12	7,50	5,22	7,70
	Conductivity [µs/cm]	·	·	·	·	·	·	2300	2350	2380	2370
	Brix	·	·	·	·	·	·	3,6	3,6	3,7	3,7
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	4,77	7,00	4,87	7,40
	Conductivity [µs/cm]	·	·	·	·	·	·	2800	2880	2840	2800
	Brix	·	·	·	·	·	·	3,6	3,5	3,6	3,5
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	$4,2x10^6$ CFU/cm^3	$3,9x10^6$ CFU/cm^3
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	$4,5x10^6$ CFU/cm^3	$4,7x10^6$ CFU/cm^3
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3

- *Lysinibacillus fusiformis* E23

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Strain belonging to the species *Lysinibacillus fusiformis* successfully multiplied in a volume of 18 liters on target media. The culture lasted for 10 days. The final culture density was 3.5 x 10^7 CFU/cm^3 in cane molasses medium to 3.7 x 10^7 CFU/cm^3 in tryptone-peptone medium. There is a marked decrease in pH values, a significant increase in ORP-Redox, and little consumption of sugars manifested by a weak decrease in Brix values. No infections with foreign microflora are observed [Table 4].

Table 4. Reactor culture parameters for *Lysinibacillus fusiformis* E23 using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Lysinibacillus fusiformis E23*											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,65	7,30	5,52	7,40
	Conductivity [µs/cm]	·	·	·	·	·	·	2400	2450	2480	2470
	Brix	·	·	·	·	·	·	3,8	3,8	3,9	3,8
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	4,88	6,80	4,80	6,60
	Conductivity [µs/cm]	·	·	·	·	·	·	2800	2880	2840	2800
	Brix	·	·	·	·	·	·	3,6	3,6	3,7	3,7
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	$3,2x10^7$ CFU/cm^3	$3,5x10^7$ CFU/cm^3
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	$3,5x10^7$ CFU/cm^3	$3,7x10^7$ CFU/cm^3
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm^3	$<1,0x10^0$ CFU/cm^3

- Levilactobacillus brevis M/495/M/17

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Strain belonging to the species *Levilactobacillus brevis* successfully multiplied in a volume of 18 litres on target media. The culture lasted for 8 days. The final culture density was 4.5 x 108 CFU/cm3 in cane molasses medium to 3.4 x 105 CFU/cm3 in tryptone-peptone medium. There is a marked decrease in pH values, a significant increase in ORP-Redox and significant consumption of sugars manifested by a significant decrease in Brix values. No infections with foreign microflora are observed [Table 5].

Table 5. Reactor culture parameters for *Levilactobacillus brevis* M/495/M/17 using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Levilactobacillus brevis* M/495/M/17											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,45	7,60	5,56	7,55
	Conductivity [µs/cm]	·	·	·	·	·	·	2000	2150	2080	2170
	Brix	·	·	·	·	·	·	3,9	3,9	3,9	3,9
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	4,15	6,70	4,20	6,65
	Conductivity [µs/cm]	·	·	·	·	·	·	2900	2980	2940	2960
	Brix	·	·	·	·	·	·	3,3	3,1	3,2	3,1
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	$1,8x10^5$ CFU/cm³	$1,1x10^4$ CFU/cm³
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	$1,7x10^8$ CFU/cm³	$1,6x10^4$ CFU/cm³
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm³	$<1,0x10^0$ CFU/cm³
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	G	G	G	G	·	·	$4,5x10^8$ CFU/cm³	$3,4x10^5$ CFU/cm³
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	$<1,0x10^0$ CFU/cm³	$<1,0x10^0$ CFU/cm³

- *Bacillus megaterium* R7

 doi:10.20944/preprints202409.2379.v1

Strain belonging to the species *Bacillus megaterium* successfully multiplied in a volume of 18 litres on target media. The culture lasted for 10 days. The final culture density was 2.7 x 10^8 CFU/cm^3 in cane molasses medium to 2.6 x 10^8 CFU/cm^3 in tryptone-peptone medium. There is a marked decrease in pH values, a slight increase in ORP-Redox for the cane molasses medium and a decrease in ORP-Redox for the tryptone-pepton medium, as well as a slight consumption of sugars manifested by a slight decrease in Brix values. No infections with foreign microflora are observed [Table 6].

Table 6. Reactor culture parameters for *Bacillus megaterium* R7 using: BPW (buffered peptone water), PCA (plate count agar), DRBC (dichloran rose bengal agar), MRS (De Man–Rogosa–Sharpe), ISA (iron sulfite agar), where symbol 'G' means growth and 'NG' means no growth.

Strain: *Bacillus megaterium* R7											
Culture stage	Parameter	Test conditions and media	Incubation	Direct culture		Non-selective multiplication		Device reading		External measurement	
				CM	TP	CM	TP	CM	TP	CM	TP
After filling the reactor with a 4% sterilised solution of cane molasses (CM) or tryptone-pepton (TP) in distilled water [about 50 µs/cm], 18 litres	pH	·	·	·	·	·	·	5,50	7,45	5,56	7,50
	Conductivity [µs/cm]	·	·	·	·	·	·	2300	2450	2350	2500
	Brix	·	·	·	·	·	·	3,7	3,7	3,8	3,8
	General contamination	BPW	Aerobic, 37°C, 24h	NG	NG	NG	NG	·	·	·	·
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	NG	NG	NG	NG	·	·	·	·
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	·	·
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	·	·
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	·	·
After inoculation and completion of culture	pH	·	·	·	·	·	·	5,15	7,00	4,20	6,65
	Conductivity [µs/cm]	·	·	·	·	·	·	2400	2080	2480	2300
	Brix	·	·	·	·	·	·	3,5	3,5	3,6	3,7
	General contamination	BPW	Aerobic, 37°C, 24h	·	·	G	G	·	·	2,6x10^6 CFU/cm^3	2,1x10^7 CFU/cm^3
	Aerobic microorganisms	PCA only/with BPW	Aerobic,30°C, 72h	G	G	G	G	·	·	2,7x10^8 CFU/cm^3	2,6x10^8 CFU/cm^3
	Fungi	DRBC only/with BPW	Aerobic, 25°C, 72h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3
	LAB	MRS only/with BPW	Anaerobic, 37°C, 72h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3
	Clostridium sp.	ISA only/with BPW	Anaerobic, 37°C, 48h	NG	NG	NG	NG	·	·	<1,0x10^0 CFU/cm^3	<1,0x10^0 CFU/cm^3

3.2. Results Based on Agar Diffusion Assay

3.2.1. Antimicrobial Activity of Bacterial Suspensions against *P. larvae* ATCC 9545

An attempt to inhibit the growth of Paenibacillus larvae ser. Eric I—a factor threatening the health of honeybee larvae—using inoculants of selected bacteria with probiotic potential under in-vitro conditions was carried out successfully. Factors capable of reducing American foulbrood pressure in bee colonies by promoting the development of a physiological colony microbiome that competes with P. larvae and blocks excessive proliferation and production of P. larvae endospores were found, and strains were selected for further selection, biocontrol of American foulbrood and development of a bioactive composition. Comparative analysis of the antimicrobial activity of culture fluids of selected bacterial species against Paenibacillus larvae ser. Eric I under in-vitro conditions showed differences between the inoculants tested and thus between the bacterial strains used, as well

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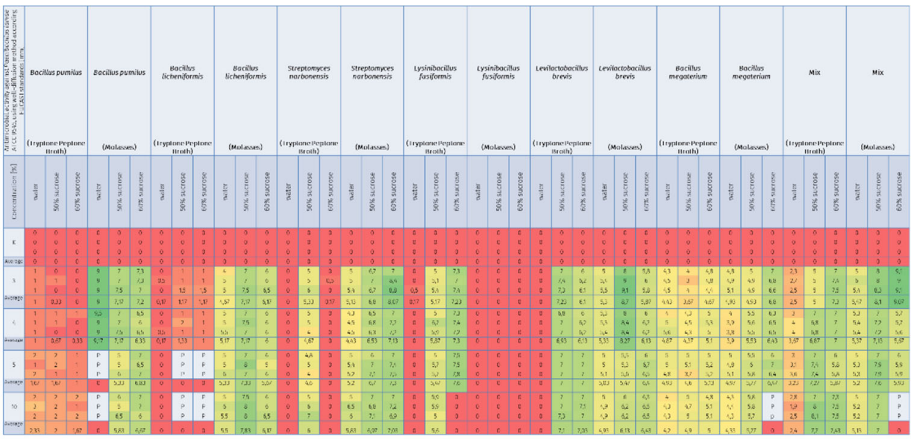
as between the media, and culture media used. Under in-vitro conditions, 9 out of 12 single inoculants tested showed suppressive effects against indicator strains belonging to the P. larvae ser. Eric I species and additionally bacterial consortia labeled as Mix [Table 7].

Table 7. Data show antimicrobial activity against *Paenibacillus larvae* ATCC 9545 (ser. Eric I), using the well-diffusion method according to EUCAST standards [mm] with heat map effect (red color means low antimicrobial activity or lack of antimicrobial properties, green color means high activity).



The greatest zone of growth inhibition of P. larvae ser. Eric I was confirmed for culture fluids of bacterial species such as B. pumilus (suspended in water, molasses culture), Mix (suspended in 60% sucrose, molasses culture), L. brevis (suspended in 50% sucrose, molasses culture), and S. narbonensis (suspended in 60% sucrose, molasses culture) qualifying these microorganisms into the group with the greatest antimicrobial activity.

The group with high antimicrobial activity was assigned to strains belonging to the species: B. megaterium (suspended in 60% sucrose, molasses culture), L. brevis (suspended in 60% sucrose and 60% sucrose, molasses culture), L. brevis (suspended in 50% sucrose and 60% sucrose, tryptone-peptone culture), L. fusiformis (suspended in 60% sucrose, tryptone-peptone culture), S. narbonensis (suspended in 50% sucrose, molasses culture), B. licheniformis (suspended in 50% sucrose, molasses culture) and B. pumilus (suspended in 50% sucrose and 60% sucrose, molasses culture).

The group with lower antimicrobial activity was assigned to strains belonging to the species: B. licheniformis (suspended in water, molasses culture), S. narbonensis (suspended in 50% sucrose, tryptone-peptone culture), S. narbonensis (suspended in water, molasses culture), L. fusiformis (suspended in 50% sucrose, tryptone-peptone culture), L. brevis (suspended in water, molasses culture), B. megaterium (suspended in all carriers, tryptone-peptone culture), B. megaterium (suspended in water and 50% sucrose, molasses culture), Mix (suspended in water, tryptone-peptone culture) and Mix (suspended in water, molasses culture).

The group with negligible antimicrobial activity included a strain belonging to the species: B. pumilus (suspended in all carriers, tryptone-peptone culture), B. licheniformis (suspended in all carriers, tryptone-peptone culture), S. narbonensis (suspended in water and 60% sucrose, tryptone-peptone culture), L. fusiformis (suspended in water, tryptone-peptone culture), L. fusiformis (suspended in all carriers, molasses culture), L. brevis (suspended in water, tryptone-peptone culture) [Table 7, Figure 1, Figure 3].

The study also showed the superiority of the 'Mix' bacterial consortium over single inoculants for biocontrol of the development of P. larvae ser. Eric I infection [Table 7, Figure 1].

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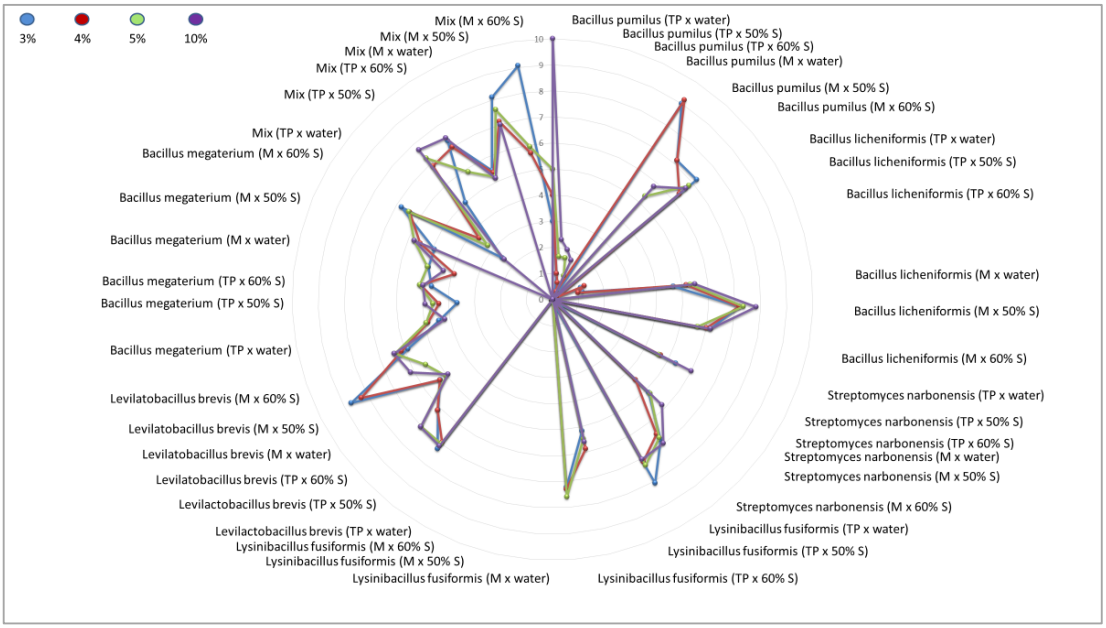

Figure 1. Radar plot illustrating the distribution of tested inoculants with antimicrobial potential against *Paenibacillus larvae* ATCC 9545 ser. Eric I using the well-diffusion method [mm] according to EUCAST standards. Volumetric concentrations of 3%, 4%, 5%, and 10% were considered, the symbol 'TP' means tryptone-peptone medium, the symbol 'M' means reed molasses medium, the symbol '50% S' means a 50% sucrose solution, the symbol '60% S' means a 60% sucrose solution, the symbol 'Mix' means a consortium of all functional strains for a given experimental system.

For some samples, bacterial overgrowth or infection of the plates marked with 'P' and 'Z' was noticed. The results of the interaction of the control samples (with water, 50% sucrose and 60% sucrose) always indicated a level of 0 mm. This confirms that the sucrose solution alone does not have an antimicrobial effect [Table 7, Figure 1].

3.2.2. Antimicrobial Activity of Bacterial Suspensions against *P. larvae* CCUG 48973

An attempt to inhibit the growth of Paenibacillus larvae ser. Eric I—a factor threatening the health of honeybee larvae—using inoculants of selected bacteria with probiotic potential under in-vitro conditions was carried out successfully. Factors capable of reducing American foulbrood pressure in bee colonies by promoting the development of a physiological colony microbiome that competes with P. larvae and blocks excessive proliferation and production of P. larvae endospores were found, and strains were selected for further selection, biocontrol of American foulbrood and development of a bioactive composition. Comparative analysis of the antimicrobial activity of culture fluids of selected bacterial species against Paenibacillus larvae ser. Eric II under in-vitro conditions showed differences between the inoculants tested and thus between the bacterial strains used, as well as between the media, and culture media used. Under in-vitro conditions, 9 out of 12 single inoculants tested showed suppressive effects against indicator strains belonging to the P. larvae ser. Eric II species and additionally bacterial consortia labeled as Mix [Table 8, Figure 3].

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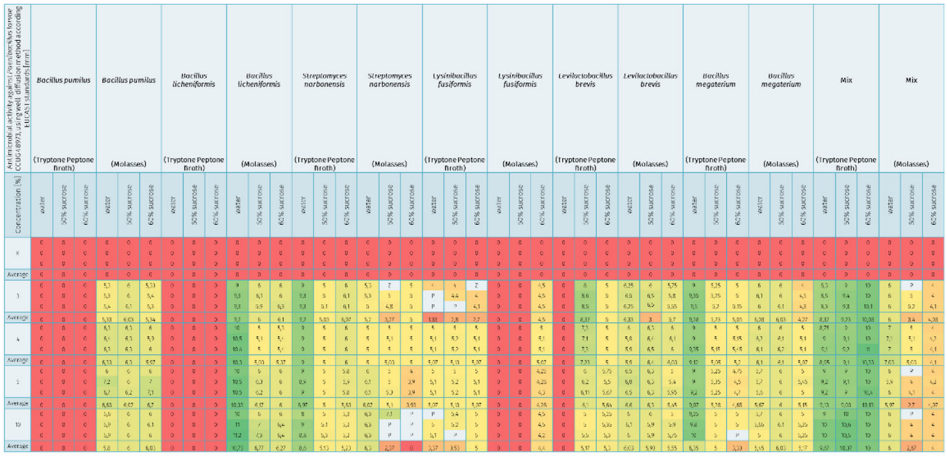
Table 8. Data show antimicrobial activity against *Paenibacillus larvae* CCUG 48973 (ser. Eric II), using the well-diffusion method according to EUCAST standards [mm] with heat map effect (red color means low antimicrobial activity or lack of antimicrobial properties, green color means high activity).



The greatest zone of growth inhibition of P. larvae ser. Eric II was confirmed for culture fluids of bacterial species such as B. licheniformis (suspended in water, molasses culture), Mix (suspended in water, 60% sucrose and 50% sucrose, tryptone-peptone culture), B. megaterium (suspended in water, tryptone-peptone culture), S. narbonensis (suspended in water, tryptone-peptone culture) qualifying these microorganisms into the group with the greatest antimicrobial activity.

The group with high antimicrobial activity was assigned to strains belonging to the species: L. brevis (suspended in 50% sucrose and 60% sucrose, tryptone-peptone culture and suspended in water, 50% sucrose, 60% sucrose, molasses culture), B. licheniformis (suspended in 50% sucrose and 60% sucrose, molasses culture), S. narbonensis (suspended in 50% sucrose and 60% sucrose, tryptone-peptone culture), S. narbonensis (suspended in water, molasses culture), B. megaterium (suspended in 50% sucrose, tryptone-peptone culture), B. megaterium (suspended in all carriers, molasses culture) and B. pumilus (suspended in water, 50% sucrose and 60% sucrose, molasses culture).

The group with lower antimicrobial activity was assigned to strains belonging to the species: S. narbonensis (suspended in 50% sucrose and 60% sucrose, molasses culture), L. fusiformis (suspended in all carriers, tryptone-peptone culture), L. fusiformis (suspended in 60% sucrose, molasses culture), B. megaterium (suspended in 60% sucrose, tryptone-peptone culture), Mix (suspended in all carriers, molasses culture).

The group with negligible antimicrobial activity included a strain belonging to the species L. fusiformis (suspended in water and 50% sucrose, molasses culture), L. brevis (suspended in water, tryptone-peptone culture), B. licheniformis (suspended in all carriers, tryptone-peptone culture) and B. pumilus (suspended in all carriers, tryptone-peptone culture) [Table 8, Figure 2, Figure 3].

The study also showed the superiority of the 'Mix' bacterial consortium over single inoculants for biocontrol of the development of P. larvae ser. Eric II infection [Table 8, Figure 2].

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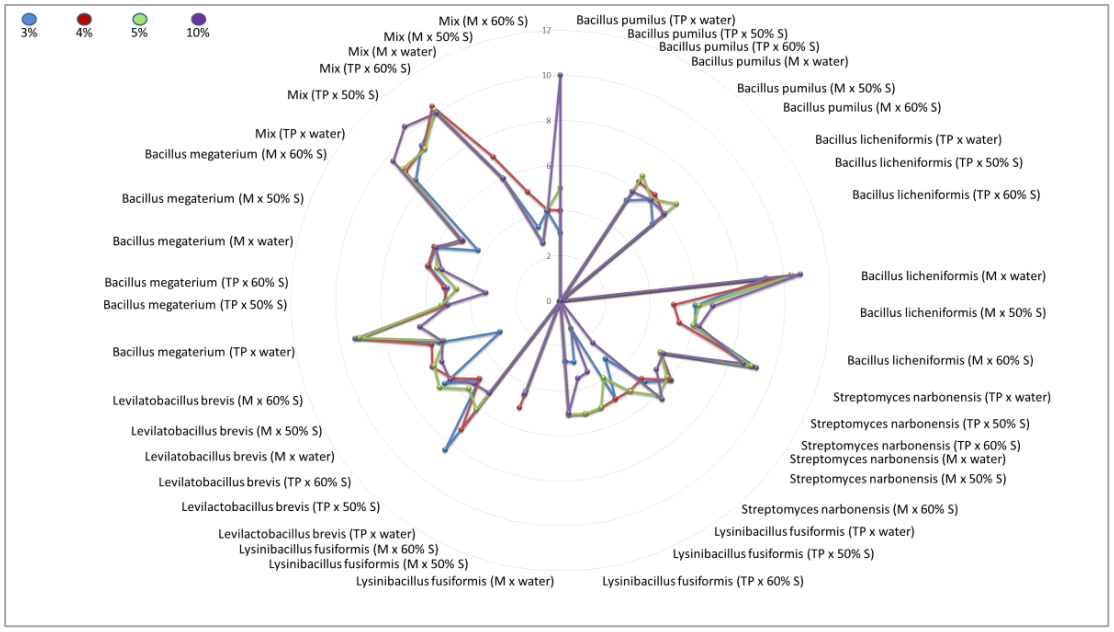
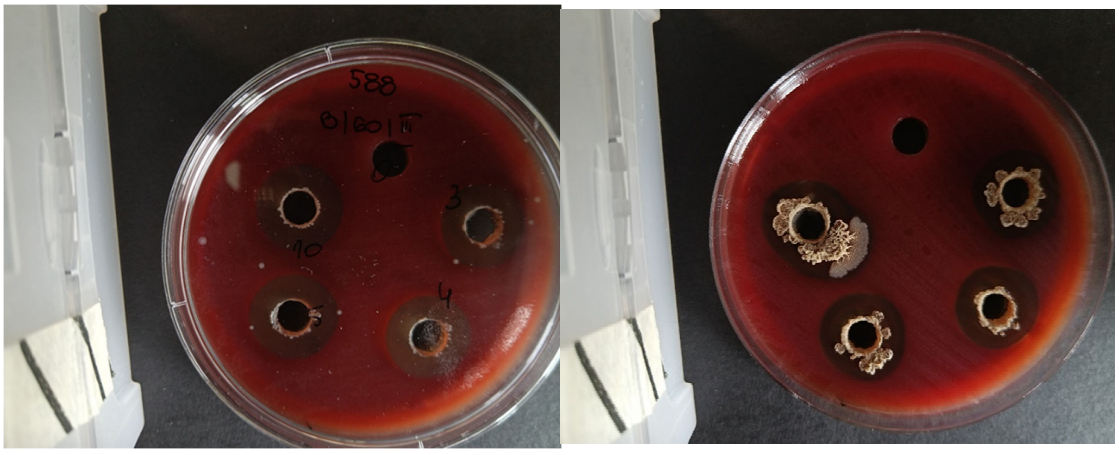


Figure 2. Radar plot illustrating the distribution of tested inoculants with antimicrobial potential against *Paenibacillus larvae* CCUG 48973 ser. Eric II uses the well-diffusion method [mm] according to EUCAST standards. Volumetric concentrations of 3%, 4%, 5%, and 10% were considered, the symbol 'TP' means tryptone-peptone medium, the symbol 'M' means reed molasses medium, the symbol '50% S' means a 50% sucrose solution, the symbol '60% S' means a 60% sucrose solution, the symbol 'Mix' means a consortium of all functional strains for a given experimental system.

For some samples, bacterial overgrowth or infection of the plates marked with 'P' and 'Z' was noticed. The results of the interaction of the control samples (with water, 50% sucrose, and 60% sucrose) always indicated a level of 0 mm. This confirms that the sucrose solution alone does not have an antimicrobial effect [Table 8, Figure 2].



(**a**) (**b**)

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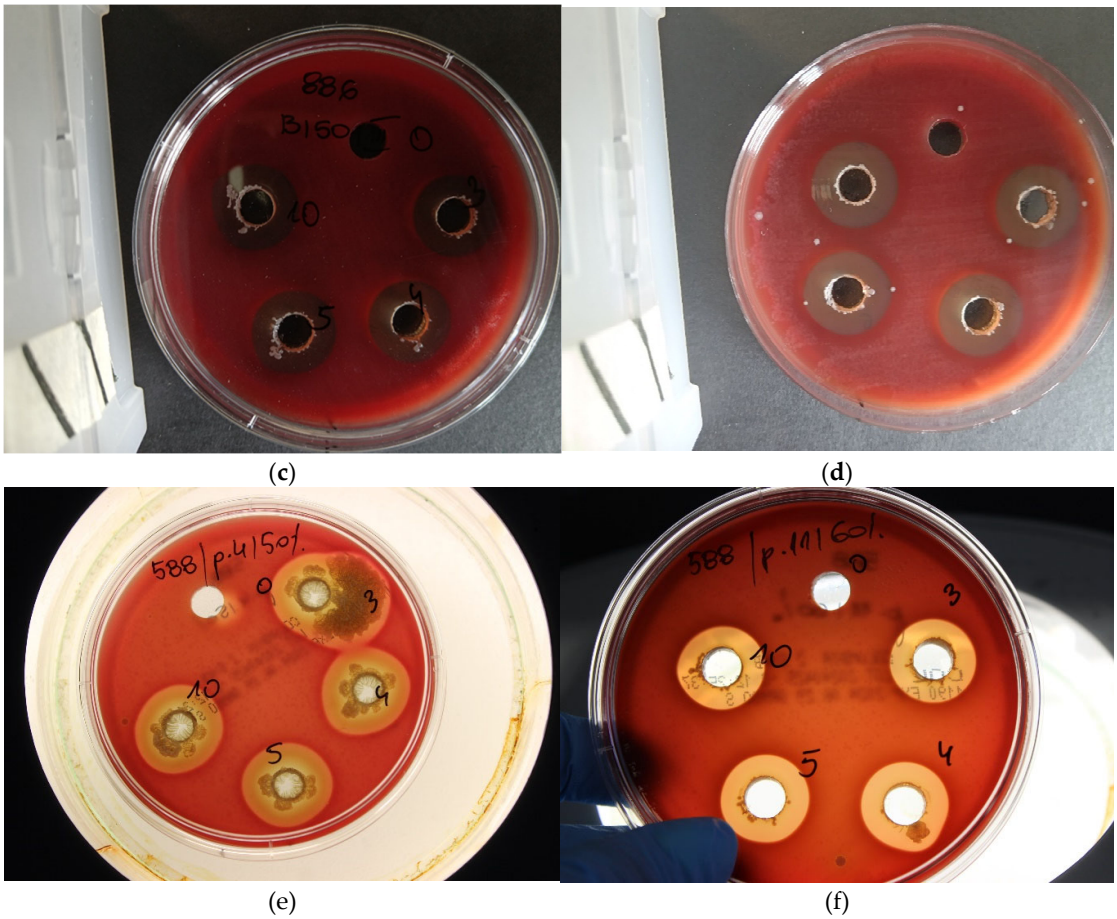

(**c**) (**d**)

(e) (f)

Figure 3. Observed 'halo' effects with a visible zone of growth inhibition against strains belonging to the P. larvae species. [**a-f**] Tests were performed using the well-diffusion method according to EUCAST guidelines with modifications due to the specific bacteria used. **[a, c, d, f]** Antimicrobial mechanisms are of interest, showing antagonism between the microorganisms used, which may include, for example, the secretion of metabiotics, i.e., metabolites and bacteriocins, called antimicrobial substances or competition for food and growth conditions. [**b, e**] Impressive bacterial overgrowth of beneficial microorganisms is also observed, which takes away the growth capacity of pathogenic cells and even leads to lysis of pathogenic cells.

3.4. Results Based on Statistical Analyses

Statistical analysis of the results obtained was performed using one-way ANOVA (Tukey's parametric post-hoc test) at a significance level of p-value < 0.05. This analysis allows the means of any number of groups to be compared with each other, showing statistically significant similarities or statistically significant differences.

It has been proven that there is a correlation and a statistically significant correlation between the microorganism used multiplied in the biotechnological fermentation process and the subsequent antimicrobial activity of the metabolites and bacteria remaining in the culture fluid. In some cases (experimental systems), there is a correlation and a statistically significant correlation between the inoculant concentration used and its subsequent antimicrobial activity. The analysis of antimicrobial activity showed statistically significant differences between the inoculants tested and therefore between the bacterial strains used and between the media, media, and indicator strains used. A one-way ANOVA analysis indicated a superior efficacy of the Mix against the pathogen analyzed. Statistical analysis (Tukey's parametric post-hoc test) indicates increased resistance of the *Paenibacillus larvae* strain ATCC 9545 Eric I to the tested agents for American foulbrood biocontrol.

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4. Discussion

In nature, the phenomena of competition and induction of antagonism between microorganisms are well known. Targeted microbial biopreparations for agriculture and wastewater treatment plants, metabolic preparations with targeted stimulatory effects in veterinary medicine, or targeted probiotics in supplementation and therapeutics with a strict therapeutic purpose and effect are of increasing interest. The approach to the gut microbiota and the environmental microbiome has changed dramatically in the last 40 years. Unfortunately, as a result of various factors, this microflora is constantly being depleted, which justifies the regular use of probiotics to restore the degraded microflora. In line with the provisions of the European Field-to-Table Strategy and the Green Deal, a reduction in the use of antibiotics in the ecosystem cycle is recommended. This is also indirectly related to increasing antibiotic resistance [70,71].

Also in the environment of the honey bee population, there is a continuous reduction of beneficial microbes. Despite prohibitions, beekeepers quite often use prohibited antimicrobial substances, including antibiotics, which then easily pass into bee products and become a real threat to consumers in the food safety chain. To overcome these problems, steps should be taken to develop a leak-proof prevention system and to implement natural product solutions, which could contain proven functional microorganisms, metabolites, or plant extracts to displace and reduce the pressure of pathogens from the bee colonies' physiological microbiome. The research undertaken in this study has achieved TRL Technology Readiness Level IV and will certainly continue to develop an effective composition to reduce bacterial superinfections caused by *Paenibacillus larvae*, together with a methodology for the application of such selected care products in beekeeping practice.

The development of American foulbrood and its milder variant, European foulbrood, caused by the bacterium *Melissococcus plutonius*, mainly occurs in stressful situations, when malnutrition and starvation occur in the bee colony. Endospores of *Paenibacillus larvae* and *M. plutonius* begin to germinate and transform into vegetative forms of the bacteria when the glucose concentration in the simple intestine of the honeybee larva falls below 2.5 %. Certainly, the conditions for the development of any bacterial or fungal brood disease are linked to a disruption of the hive microflora, which, as a result of undetermined factors, has been reduced to such an extent that the spread of American foulbrood bacteria occurs at an extremely fast rate, leading to an exponential logarithmic growth of vegetative forms. American and European foulbrood bacteria are proteolytic pathogens, meaning that they rapidly lead to cytolysis and histolysis of the bee larvae. When the hive has a shaky, impoverished microbiome, even the smallest number of spores delivered to the hive with frames or through bee trophallaxis can lead to the expansion and domination of the hive environment by *P. larvae* [72–74].

Beekeepers' use of mite killers to reduce the population of the bee tormentor *Varroa destructor* also has a large impact on the significant reduction of the beneficial hive microflora. Such agents include acaricides, pyrethroids, organic acids, thymol, or organophosphate insecticides [75].

The problem is global and also affects regions where antibiotic treatment is allowed. The pharmacokinetics of an antibiotic usually ends when one or, less frequently, two of the active substances are depleted or degraded, causing bacteria to rapidly acquire resistance to the active substance. In the case of probiotics, there is a specific fusion of many different biologically active substances with a complex structure and diverse mechanisms of action, which means that the bacterial cell is not able to produce as many defense mechanisms and provide resistance. Probiotic bacteria secrete a variety of phenol acids, flavonoid compounds, phenolic compounds, bacteriocins, antibiotics, organic acids, bioflavonoids, and biologically active compounds [76–78].

Researchers searching for microbial antagonistic agents for American foulbrood biocontrol are most often limited to testing strains from the genera *Lactobacillus* and *Bifidobacterium*. Studies in which bacteria obtained from healthy bee colonies are isolated are also encountered. These are specific microorganisms derived from the microbiome of bee colonies and usually involve the genera *Bacillus sp., Streptococcus, Candida sp., Saccharomyces, Pseudobacterium, Fructobacillus, Parasaccharibacter, Ochrabactum, Acinetobacter, Stenotrophomonas, Gilliamella, Snodgrassella, Actinomadura, Streptomyces, Apilactobacillus, Bombilactobacillus* and many others. As there are between 6,000 and 8,000 different

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microbial strains in the bee microbiome it seems nothing new or harmful to administer specific probiotics and supplement the microflora. In human medicine, the effect of so-called probiotic interventions and the administration of specific strains to support, for example, diabetes, cancer, or obesity, thus modifying the diseased microbiome, is increasingly being studied [79–82].

The results from the comparative analysis of the antimicrobial activity of culture fluids of selected probiotic bacterial species against *Paenibacillus larvae* ser. Eric I and II under in-vitro conditions are consistent with literature data and tests evaluated by E. Forsgren, S. Lamei, and T. Olofsson [83–85]

High efficacy in antimicrobial activity was also achieved in a 2006 study conducted by Alippi and Reynaldi, confirming high antimicrobial activity for *Bacillus pumilus, Bacillus licheniformis,* and *Bacillus megaterium* against *P. larvae* ATCC 9545 [86].

Audisio et al. described the effective combination of a *Bacillus subtilis* strain with a plant extract against *P. larvae* highlighting the high utility of *Bacillus spp.* [87].

Also Bartel and Alippi in 2018 identified natural antagonists of *P. larvae* by selecting strains of *Bacillus* species and related genera producing a broad range of antimicrobial compounds, with activity against bacteria and fungi that include peptides, lipopeptides, bacteriocins, and bacteriocin-like inhibitory substances. By using biological tools, they evaluated the antagonistic activity of 34 bacterial strains against *Paenibacillus larvae* and *Ascosphaera apis*, the causal agents of American Foulbrood and Chalkbrood diseases of honey bee larvae, respectively. In the work in question, a strong bactericidal effect attributed to a strain of *Bacillus megaterium* and *Bacillus licheniformis* was demonstrated. Similarly, in studies by other researchers, *B. megaterium* showed strong antimicrobial activity due to the production of megacins by *Bacillus megaterium* and also lichenin and lichenicidin produced by *Bacillus licheniformis* [88].

Studies by other authors have documented that *Bacillus licheniformis* and another strain tested in the experiment reduced the mortality of American foulbrood-infected larvae. Treatments showed positive effects and reduced mortality [89].

Many studies by other authors confirm the efficacy of the bacterial species analyzed in reducing American foulbrood. This demonstrates the pertinent and successful selection of bacterial strains that can find application in the prevention of this disease. In other studies, *B. licheniformis* and *B. subtilis* also showed antimicrobial activity against *P. larvae* isolated from the bee environment in Saudi Arabia [90].

There are no sources that report what antagonistic effect *Streptomyces narbonensis* species may have. Korean researchers demonstrated that isolates extracted from forest soil, also identified as human pathogens, containing mainly strains of the genus *Streptomyces* showed strong pressure and antimicrobial activity against *Paenibacillus larvae* isolates supporting the theory that the source of the search for microbial agents need not only be the healthy bee microbiome but also other sources and natural resources [91].

Bacterial metabolites are reported in great detail by Adrian A. Pinto-Tomas and co-workers, who isolated *Actinobacteria* in the genus *Streptomyces* from foraging bees, and especially common in pollen stores. One strain, isolated from pollen stores, exhibited pronounced inhibitory activity against *Paenibacillus larvae* [92].

Q. Jamal, on the other hand, describes the widespread use of bacteria of the genus *Lysinibacillus.* Moreover, some *Lysinibacillus* species have antimicrobial potential due to bacteriocins, peptide antibiotics, and other therapeutic molecules. Therefore, this review will explore the biological disease-fighting, food preservation, therapeutic, plant growth-promoting, bioremediation, and entomopathogenic potentials of the genus *Lysinibacillus* [93].

This shows a well-chosen vector of search and selection of strains for the work in question. Good probiotic properties, high survival rates, and origin from the hive microbiome are reported by Polish researchers in the context of *B. pumilus,* and *B. licheniformis* which again shows the correct selection in microbial agents for biocontrol of American foulbrood. It is therefore confirmed that *B. pumilus* and *B. licheniformis* species also occur in the hive environment [94]

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Levilactobacillus brevis also shows the ability to reduce the biofilm formation of *P. larvae* isolated from honey bee guts or fresh pollen samples, as indicated by the mechanism of action of this species [95].

In a study by Polish scientists, *Levilactobacillus brevis* B50 increased the expression of pattern recognition receptors and genes encoding antimicrobial peptides (defensin-1 and abaecin [96]. The LAB isolated from the honey bee gut has been demonstrated to be helpful for the inhibition of *P. larvae*. The antibiotic action of *L. brevis* is also based on its secretion of organic acids [97,98].

5. Conclusions

Using lactic acid bacteria (LAB) as an alternative to chemical treatments is a promising novel technique for tackling honey bee diseases and improving their immunity. The concept of reducing bacterial superinfections should move towards strict prevention using biosecurity to improve the welfare of honeybee colonies. It is highly advisable that in the case of developing bacterial superinfections with *P. larvae*, the legislative authorities, through veterinary entities, authorize specific measures consisting of the implementation of probiotic interventions using inoculants of tested bacteria with proven beneficial effects on bees. Such a strategy is illustrated by results from numerous scientific reports.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table 7: Data show antimicrobial activity against *Paenibacillus larvae* ATCC 9545 (ser. Eric I), using well-diffusion method according to EUCAST standards [mm] with heat map effect (red color means low antimicrobial activity or lack of antimicrobial properties, green color means high activity); Figure 1: Radar plot illustrating the distribution of tested inoculants with antimicrobial potential against *Paenibacillus larvae* ATCC 9545 ser. Eric I using the well-diffusion method [mm] according to EUCAST standards. Volumetric concentrations of 3%, 4%, 5%, and 10% were considered, the symbol 'TP' means tryptone-peptone medium, the symbol 'M' means reed molasses medium, the symbol '50% S' means a 50% sucrose solution, the symbol '60% S' means a 60% sucrose solution, the symbol 'Mix' means a consortium of all functional strains for a given experimental system; Table 8: Data show antimicrobial activity against *Paenibacillus larvae* CCUG 48973 (ser. Eric II), using well-diffusion method according to EUCAST standards [mm] with heat map effect (red color means low antimicrobial activity or lack of antimicrobial properties, green color means high activity); Figure 2: Radar plot illustrating the distribution of tested inoculants with antimicrobial potential against *Paenibacillus larvae* CCUG 48973 ser. Eric II uses the well-diffusion method [mm] according to EUCAST standards. Volumetric concentrations of 3%, 4%, 5%, and 10% were considered, the symbol 'TP' means tryptone-peptone medium, the symbol 'M' means reed molasses medium, the symbol '50% S' means a 50% sucrose solution, the symbol '60% S' means a 60% sucrose solution, the symbol 'Mix' means a consortium of all functional strains for a given experimental system; Figure 3: Observed 'halo' effects with a visible zone of growth inhibition against strains belonging to the *P. larvae* species. [a-f] Tests were performed using the well-diffusion method according to EUCAST guidelines with modifications due to the specific bacteria used. [a, c, d, f] Antimicrobial mechanisms are of interest, showing antagonism between the microorganisms used, which may include, for example, the secretion of metabiotics, i.e., metabolites and bacteriocins, called anti-microbial substances or competition for food and growth conditions. [b, e] Impressive bacterial overgrowth of beneficial microorganisms is also observed, which takes away the growth capacity of pathogenic cells and even leads to lysis of pathogenic cells.

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