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POSSIBILITY OF ENRICHMENT PROBIOTICS FOR RUMINANTS IN PLANT OILS FOR IMPROVING THEIR ACTIVITY AGAINS HARMFUL BACTERIA

Summary

Among proposed alternatives to antibiotic growth promoters (AGPs), most effective in improving of efficiency of farming and having positive impact on animal welfare are probiotic preparations containing selected strains of lactic acid bacteria (LAB). It is known that essential oils exert ability to inhibit pathogenic bacteria. The aim of this study was to assess the possibility of enrichment of probiotic preparations for animals in selected substances of vegetable origin, in order to increase the effectiveness of antimicrobial activity LAB directed against pathogenic microorganisms. The impact of three selected essential oils (thyme, sage and peppermint) on the capacity to growth, viability and antibacterial activity of probiotic or potentially probiotic strains of lactic acid bacteria (LAB) was evaluated. The assessment of the antibacterial activity of the LAB was conducted in relation to the bacteria strains pathogenic for animals (indicatory strains). The results show that the reaction of lactic acid bacteria strains for the presence of vegetable oils is very individual, depending on both the strain and the type of oil. All the tested plant's oils inhibited the growth of strains of lactic acid bacteria. The minimum inhibitory concentration of essential oils varied. In the case of peppermint oil and thyme oil LAB growth inhibition was observed at a concentration of 0.1% (v/v) and in relation to the sage oil this effect was found with 0.2 %.(v/v) average in medium. The viability of the tested LAB was observed in media containing higher concentrations up to several per cent of essential oils. In the presence of oils in concentrations affecting growth of LAB strains antimicrobial activity against pathogenic bacterial species was not observed. The addition of essential oils did not increase the antimicrobial activity of lactic acid bacteria. It was observed that some of indicatory pathogenic bacteria strains were resistant to higher dilution of oils than LAB. There was no synergistic interaction found between LAB and essential oils what suggest the possibility of using essential oils with LAB only to increase the flavor qualities of preparation which will contribute to a better feed intake by the farm animals.
***Key words**: essential oils, probiotics for ruminants, LAB*

OCENA MOŻLIWOŚCI WZBOGACANIA PREPARATÓW PROBIOTYCZNYCH DLA PRZEŻUWACZY W SUBSTANCJE ROŚLINNE

Streszczenie

W grupie proponowanych zamienników antybiotykowych stymulatorów wzrostu (ASW) największą skutecznością działania w podwyższaniu efektywności hodowli i pozytywnym wpływaniu na dobrostan zwierzą charakteryzują się preparaty probiotyczne, zawierające wyselekcjonowane szczepy bakterii fermentacji mlekowej (LAB). Wiadomo również, że roślinne olejki hamują wzrost niektórych bakterii patogennych. Celem pracy była ocena możliwości wzbogacenia preparatów probiotycznych dla zwierząt w wybrane substancje pochodzenia roślinnego, tak aby zwiększyć skuteczność antymikrobiologicznego oddziaływania LAB, skierowaną przeciw drobnoustrojom patogennym. Zbadano wpływ trzech wybranych olejków eterycznych (tymiankowego, szałwiowego i miętowego) na wzrost, przeżywalność i aktywność antybakteryjną probiotycznych lub potencjalnie probiotycznych szczepów bakterii fermentacji mlekowej (LAB). Ocenę aktywności antybakteryjnej lab przeprowadzono w odniesieniu do panelu szczepów patogennych dla zwierząt hodowlanych. Otrzymane wyniki wskazują, że reakcja szczepów bakterii fermentacji mlekowej na obecność olejków roślinnych jest bardzo indywidualna, zależna zarówno do szczepu jak i rodzaju olejku. Wszystkie badane olejki eteryczne hamowały wzrost szczepów bakterii fermentacji mlekowej, jednakże stężenia olejków hamujące wzrost były zróżnicowane. W przypadku olejku miętowego i tymiankowego hamowanie wzrostu LAB obserwowano już przy stężeniu 0,1%, a w przypadku olejku szałwiowego taki efekt stwierdzono przy jego obecności w podłożu w stężeniu 0,2%. Natomiast przeżywalność badanych LAB obserwowano w podłożach zawierających wysokie, sięgające kilku procent stężeniach olejków eterycznych.. Aktywność antybakteryjną szczepów LAB wobec gatunków bakterii patogennych obserwowano w obecności nie wpływających na wzrost stężeń olejków. Dodatek olejków eterycznych nie powodował zwiększenia aktywności antymikrobiologicznej bakterii fermentacji mlekowej. Wykazano możliwość stosowania olejków eterycznych, w odpowiednich stężeniach, jako jednego z komponentów wieloskładnikowych preparatów probiotycznch przeznaczonych dla zwierząt. Dodatek olejku eterycznego miałby na celu uatrakcyjnienie walorów zapachowych preparatu a przez to jego lepsze pobieranie przez zwierzęta hodowlane.
***Słowa kluczowe**: olejki eteryczne, probiotyki dla przeżuwaczy, LAB*

1. Introduction

The studies which have been conducted all over the world through recent years, have revealed that among proposed alternatives to antibiotic growth promoters (AGPs) the most effective ones are probiotic preparations containing selected strains of lactic acid bacteria (LAB). They limit mortality and have positive impact on animal welfare and efficiency of farming. Probiotic properties of LAB are the result of their specific abilities including antagonistic activity against enteric pathogens and resistance to the acidic environment of the stomach and bile salts. Probiotics are characterized by resistance to acids and other antagonistic compounds synthesized by the intestinal microorganisms. Antimicrobial activity of the LAB, including probiotic bacteria is associated with the ability to synthesize antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins [1, 2, 22].

Proposed replacements of AGPs are also essential oils which are multi-component mixtures of secondary volatile metabolites of plants. Essential oils are characterized by antibacterial, anti-inflammatory and antioxidant activities. They also regulate metabolism and stimulate the immune system of animals. Essential oils, as ASWreplacements, were administered during in vivo studies to the different groups of farm animals - mainly poultry and pigs, but also cows, and even fishes. Polish research results on herbal preparations, replacing feed antibiotics have shown that the efficacy of herbal remedies is only marginally lower (in some cases even equal) than the efficacy of AGPs in reducing the mortality of young animals, especially poultry and piglets [15, 23]. Santos [25] proved a positive effect of the Agolin Ruminant preparation containing eugenol, essential oils of geranium and coriander on improving the quality of milk and general health of dairy cows.

The growing interest in use of essential oils as antimicrobial agent in food and feed is a consequence of the consumer’s demand for natural "bio-preservatives”- plant derived substances with biological activity (antimicrobial properties), safe for humans and environment-friendly. Essential oils are allowed to use in organic farming because they enable to obtain natural and safe products while avoiding the addition of chemical preservatives and antibiotics.

Antibacterial and antifungal properties of essential oil are related to their unique chemical composition i.e. the presence of volatile products of an aromatic plants (oft herbs and spices). It has been detected that essential oils contain from a few to a few hundred, naturally synthesized by plants, chemical compounds: phenols, terpenoids, alcohols, aldehydes, ketones and others [5, 14, 18, 19, 24, 27]. According to many papers the strongest impact on microorganisms have essential oils containing phenols (thymol, carvacrol, eugenol), some of them are GRAS status (classified as Generally Recognized as Save) [5, 18, 19]. These compounds include essential oils of thyme, savory, oregano and clove oils. In many cases, synergistic interactions between the components of essential oils are observed that antimicrobial activity of the mixture is stronger than the individual components [9, 12]. Considering safety of essential oils, there is no evidence for developing resistance by microorganisms in contact with them or transmitting resistance by the horizontal gene transfer [27]. Furthermore, essential oils do not cumulate in the human and animals body, and in contrast to antibiotics, they are easily biodegradable [14, 27].

The sensitivity of the microorganism to a particular essential oil depend on the type of essential oil and its concentration [3, 13, 14, 26]. The results of the evaluation of the antimicrobial properties of essential oils are also influenced by such factors as the type of extract, species, and even strain of tested microorganisms, methods and test conditions [14].

The important parameters evaluated in studies are MIC (minimum inhibitory concentration) and MBC (maximal tolerated concentration) [6,10]. Samie [24] investigated a large panel of microorganisms belonging to the genus *Acinetobacter*, *Bacillus*, *Escherichia*, *Klebsiella*, *Serratia*, *Staphylococcus*, *Streptococcus* (including both strains from the collection of microorganisms and clinical / environmental isolates) for susceptibility to essential oils originating from endemic African plants and evaluated a large range of MIC values - from 0.95 to 7.5 mg/ml. Similarly, large spread of MIC values results was obtained by Delaquis [7] in studies of antimicrobial properties of essential oils of eucalyptus, fennel and coriander directed against strains of *Listeria monocytogenes*. The important parameter is also the time of contact between oils and bacteria which in case of oils is rather short.

Most studies show that Gram-negative bacteria are more resistant to antimicrobial compounds of essential oils (due to the different structure cellular membrane) than Gram-positive bacteria, but this is not a strict rule, and depends on the strain of bacteria [3, 7, 8, 20]. For example, in Hać-Szymańczuk et all studies [11] greater sensitivity to the presence of essential oils and extracts of rosemary was observed in case of Gram-positive than Gram–negative bacteria. This might suggest a high sensitivity of lactic acid bacteria to the presence of oils, as belonging to the group G+. However research work attempting to enhance the effect of the probiotic *Lactobacillus fermentum* on poultry by the addition of oregano and thyme essential oils showed beneficial impact of the essential oil presence on antibacterial activity of probiotic bacteria *L. fermentum* against *Salmonella* strain [16].

So far, studies on the antimicrobial properties of essential oils were focused mainly on determining their effects on pathogens and potentially harmful bacteria, merely a small number of studies concerned LAB. Perhaps the probiotic preparations and plant’s oils simultaneous use in feeding young animals will increase the effectiveness of both substances, without a significant reduction in the number of lactic acid bacteria. Testing the application of essential oils in the food and feed should consider not only their antimicrobial activity but also the organoleptic aspects, so that the concentration of essential oil, having respective antibacterial effect, does not cause such product to be organoleptic unacceptable to the animals [20]. The possibility of combined use of essential oils and LAB in preparations for animal requires defining the LAB resistance to oils. Additional premise of undertaking a research are the results of experiments indicating the individual nature of the resistance/susceptibility of bacteria to essential oils. The paper attempts to answer the question whether it is possible to enrich the probiotic preparations for animals in selected spice oils.

2. Aim of the study

The aim of this study was to assess the possibility of enrichment of probiotic preparations for animals developed in the ZF IBPRS in selected substances of vegetable origin, in order to increase the effectiveness of their antimicrobial effects directed against pathogenic microorganisms.

3. Material and methods

In the study following strains of lactic acid bacteria were used: Lactobacillus casei KKP 824, *Lactococcus lactis ssp lactis* KKP 835, *Lactobacillus delbrueckii* KKP 838, *Pediococcus sp.* KKP 841 These strains belong to the collection of Industrial Culture IBPRS (KKP) and are stored in liquid nitrogen. Lactobacillus casei 824 KKP is a probiotic strain, included in the commercial IBPRS Probiomix C, designed for calves. Strains *Lactococcus lactis ssp lactis* KKP 835, *Lactobacillus delbrueckii* KKP 838, *Pediococcus sp*. KKP 841, have been isolated from raw milk [4]. This LAB strains were identified based on 16S rDNA sequence and defined as potentially probiotic. Strains were maintained in liquid nitrogen atmosphere in bullion medium with 10% of glycerol.

Inoculum for each test was the second passage cultures grown for 24 h, after the transfer of bacteria from liquid nitrogen to MRS medium (Merck). In order to obtain initial number of bacteria cells in cultures on the level approximately 1 x 10^6 CFU/ml appropriate dilutions were used. Controls were cultures LAB strains in MRS without the addition of oils. The growth capacity of LAB (lactic acid bacteria) in the presents of selected plant's oils were assessed in 24 - hour cultures, at 37° C, in MRS liquid medium with the addition of thyme, mint or sage oils in adequate amount to achieve dilution oil from 0.025% up to 8% (v/v), according to bacterial resistance.

The antimicrobial activity of LAB in the presence of plant's oils were evaluated using the agar diffusion method against strains: *Salmonella* serotype DO, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Virchow, *Salmonella enterica* serotype Saintpaul, *Salmonella enterica* serotype enteritidis, *Listeria monocytogenes*, *Escherichia coli* hemolytic strain [21]. Test (indicatory) strains were obtained from PIWET PIB (National Veterinary Research Institute) in Pulawy. After 24 incubation in presence of oils the LAB cultures were centrifuged and samples of supernatant were spread on the solidified MRS soft agar (0.75% agar), inoculated with test/indicator bacteria strains. Bacterial cultures without essential oils in MRS were used as controls. The results were assessed after 24 hours incubation at room temperature as the diameter of inhibition zone of pathogen bacteria. Antibacterial activity oils against the pathogenic strains of bacteria were evaluated as resistance/viability after culture with plan's oils in concentration 0,1-0,6% (v/v).

There were used thyme, mint and sage oils in the form of 100% aqueous distillates, distributed by Green Village which declared the quality of oils in accordance with ISO standards, Polish and German Pharmacopoeia and certificated by the National Institute of Hygiene in Warsaw [www.greenvillage.pl].

The number of lactic acid bacteria was determined by culture method plates on solid MRS with addition bromocresol purple. The number of pathogenic bacteria was determined by spread on nutrient broth agar and on solid Rambach agar plates (Merck). Effect of essential oils on the amount of lactic acid, synthesized by the LAB was assessed in the effluent after separation bacterial culture with the addition of essential oils, using the enzyme assays test Boehringer-Mannheim.

Each assay was performed on two separate experimental runs and all analyses were carried out in duplicate. The data were reported as means ±standard deviation.

4. Results and discussion

The capability of the growth and viability of the tested LAB strains in the presence of plant oils are shown in Figure 1. The results indicate that the susceptibility of lactic acid bacteria strains on the presence of plant's oils is very individual, depending on both the strain and the type of oil. Low concentrations of all tested oils in the medium (0.05%) do not negatively affect the growth of the LAB. Thyme oil seems to exhibit the strongest negative impact on all tested bacteria strains, a slight increase in the concentration of thyme oil to 0.1% in MRS resulted in reduction of the bacteria cells number below the level of the initial LAB (10^6 CFU/ml). These results were in accordance with the studies showing the strongest bactericidal activity of oils containing phenols, since the main components of thyme oil are phenols: thymol (20-40%), carvacrol and alcohols [14,17]. Only *Lactobacillus lactis ssp. lactis* KKP 835 of the investigated LAB was characterized by their ability to growth under these conditions. The MTC (the highest concentration of thyme oil, which did not affect the bacterial growth) with respect to this strain was 0,1% (v/v). This strain of bacteria seems to be the most resistant to presence of plant's oils.

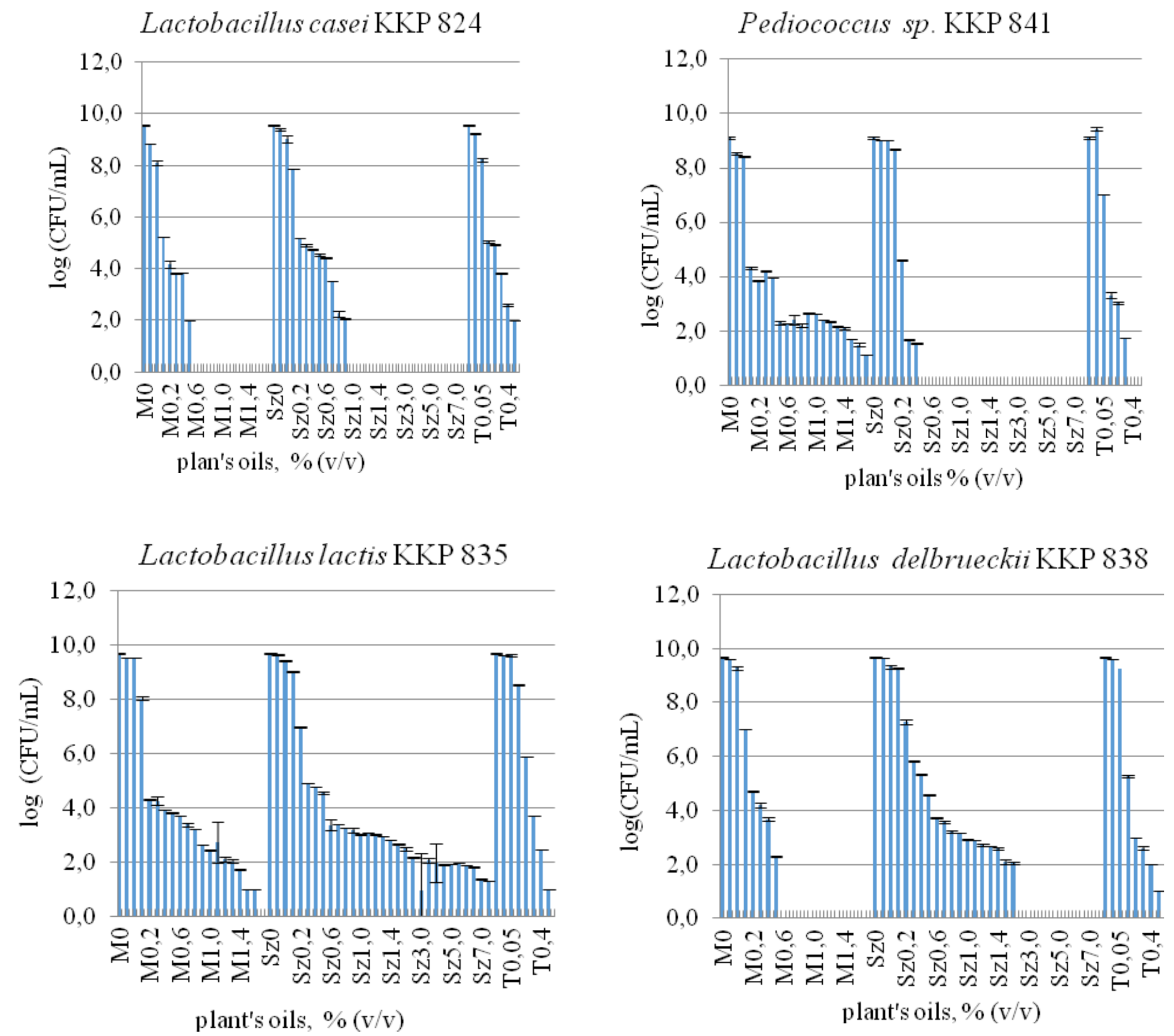
Slight different level of bacteria sensitivity was observed in the case of peppermint oil. The tested LAB strains were capable to growth at peppermint oil concentration 0,1% (the MTC). Unexpectedly *Pedicoccus* sp.KKP 841 strain, that was found to be the most sensitive, characterized resistance (viability) in relatively high concentration of peppermint oil i.e. 2%.

Peppermint oil consists among other substances of the menthol and menthol esters, ketones, menthofuran, monoterpenes and terpene oxides. *Lactobacillus delbrueckii* KKP 838 was the only one tested LAB strains exhibited the ability to grow in the presence of the sage essential oil at concentration of 2% (v/v). This observation confirmed that susceptibility of LAB to essential oils is very diverse and depends on oils and bacteria strains [19,24,26]. In the sage essential oil were detected inter alia: α - pinene, cineol, α - and β - thujone, borneol and others [17].

Evaluation of antimicrobial activity of the tested bacterial strains cultured in presence essential oils was performed using only samples in which the growth of LAB was observed. The concentrations of essential oils not affecting the growth of the cultures did not exceed 0.05% for thyme and 0.1% for mint and sage. Antimicrobial activity of lactic acid bacteria cultured in media supplemented with essential oils is shown in Table 1.

The results of antibacterial activity of LAB cultured in presence of essential oils indicated that activity against pathogenic bacteria strains is correlated with the capacity to growth. There were also evaluated the capacity to lactic acid production of LAB that rapidly decreased in presence of plant's oils in concentration higher then 0,05% (v/v) (results are not shown). Exclusively in the case of growth of LAB are detected antibacterial activity against pathogens. No effect of accumulated activity of LAB and essential oils on pathogenic bacteria was found.

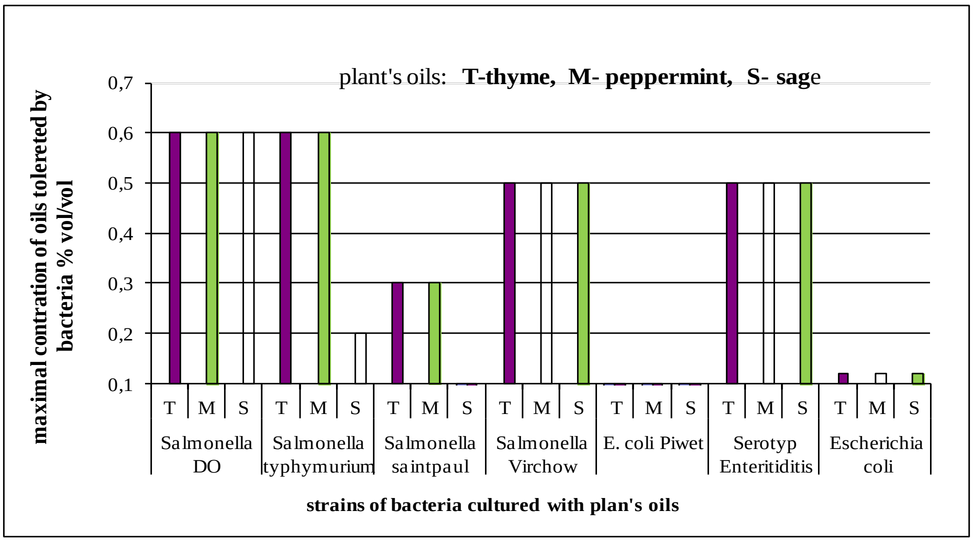
The influence of plant's oils on the viability of indicatory strains, pathogenic for animals (mainly poultry) are shown in figure 2.



Source: Own work / Źródło: opracowanie własne

Fig. 1. The growth capacity /viability of lactic acid bacteria strains in the presence of thyme (T), sage (Sz) and peppermint (M) oil in culture medium

Rys. 1. Zdolność wzrostu / przeżywalność szczepów bakterii fermentacji mlekowej w obecności w podłożu hodowlanym olejków: tymiankowego (T), szałwiowego (Sz) I miętowego (M)



Source: Own work / Źródło: opracowanie własne

Fig. 2. The influence of plant oils on the viability of indicatory strains

Rys. 2. Wpływ olejków roślinnych na przeżywalność bakterii wskaźnikowych

Table 1. Antimicrobial activity of lactic acid bacteria in presence of plan’s oil against indicatory bacteria
Tab. 1. Aktywność antymikrobiologiczna bakterii fermentacji mlekowej wobec bakterii wskaźnikowych w obecności olejków roślinnych

Strain of bacteria	Oil % vol/vol	Indicatory bacteria strains, inhibition zones (mm)						
		S. serotyp DO	*S.* serotyp typhimurium	*S. enterica* serotyp Virchow	*S. enterica* serotype Saintpaul	*S. enterica* serotyp Enteritidis	*Listeria monocytogenes*	*Escherichia coli*
L casei KKP 824	Thyme 0,025%	11,0±0,71	nd	10,5 ±1,41	8,0±0,71	6,0 ±0,71	7,0 ±0,71	9,0 ±0,71
	Thyme 0,05%	8,0 ±0,71	nd	8,0 ±0,71	9,0 ±0,71	nd	7,0 ±0,71	nd
	Mint 0,025%	7,0 ±0,71	nd	9,0 ±0,71	7,0 ±0,71	nd	6,5 ±1,41	12 ±0,71
	Mint 0,05%	nd	nd	nd	nd	nd	nd	nd
	Sage 0,05%	nd	nd	nd	nd	nd	nd	nd
	Sage 0,1%	6,5±1,41	nd	5,0 ±0,71	nd	nd	7,0 ±0,71	nd
	K	10,5 ±1,41	nd	11,0±0,71	8,0 ±0,71	5,0 ±0,71	8,0 ±0,71	8,5 ±1,41
L. delbruecki KKP 838	Thyme 0,025%	11,0± 0,71	nd	12,5 ±1,41	9,5 ±1,41	10,0 ±0,71	9,0 ±0,71	10 ±0,71
	Thyme 0,05%	11,0 ±0,71	nd	6,0 ±0,71	6,5 ±1,41	9,0 ±0,71	7,5 ±1,41	-
	Mint 0,05%	13,0 ±0,71	nd	11,0 ±0,71	11,0± 0,71	7,0 ±0,71	9,0 ±0,71	9,0 ±0,71
	Mint 0,1%	nd	nd	nd	nd	nd	nd	nd
	Sage 0,05%	11,0± 0,71	nd	7,5 ±1,41	7,0 ±0,71	7,5 ±1,41	5,0 ±0,71	6,0 ±0,71
	Sage 0,1%	12,5±1,41	nd	8,0 ±0,71	8,0 ±0,71	7,5 ±1,41	7,0 ±0,71	7,0 ±0,71
	K	12,0± 0,71	nd	8,0 ±0,71	7,0 ±0,71	nd	11,5 ±1,41	
L. lactis KKP 835	Thyme 0,025%	12,0± 0,71	nd	15,0± 0,71	10,0 ±0,71	6,5 ±1,41	10,0 ±0,71	13,0 ±0,71
	Thyme 0,05%	12,5±1,41	nd	13,0 ± 0,71	8,5 ±1,41	6,0 ±0,71	10,0 ±0,71	11,5 ±1,41
	Mint 0,05%	11,0 ±0,71	nd	12,5 ± 1,41	8,0 ± 0,71	5,0 ±0,71	9,0 ±0,71	14,0 ±0,71
	Mint 0,1%	7,0 ±0,71	nd	5,0 ±0,71	6,5 ±1,41	nd	7,0 ±0,71	sd
	Sage 0,05%	10,0± 0,71	nd	10 ±0,71	7,5 ±1,41	6,0 ±0,71	10,0 ±0,71	8,0 ±0,71
	Sage 0,1%	9,5±1,41	nd	12± 0,71	8,0 ±0,71	5,0 ±0,71	9,5 ±1,41	9,0 ±0,71
	K	12,0±0,71	nd	11,5 ±1,41	8,0 ±0,71	6,0 ±0,71	13,0± 1,41	15,0 ±0,71
Pediococcus sp. KKP 841	Thyme 0,025%	nd	8,0 ±0,71	9,0 ±0,71	9,0 ±0,71	nd	7,5 ±1,41	8,0 ±0,71
	Thyme 0,05%	nd	6 ,0±0,71	7,0 ±0,71	7,0 ±0,71	nd	5,5 ±1,41	5,5 ±1,41
	Mint 0,025%	nd	7,5 ±1,41	7,0 ±0,71	8,5 ±1,41	nd	7,0 ±0,71	,09 ±0,71
	Mint 0,05%	nd	nd	nd	nd	nd	nd	nd
	Sage 0,025%	nd	7,0 ±0,71	6,5 ±1,41	10,0 ±0,71	nd	6,0 ±0,71	7,0 ±0,71
	Sage 0,05%	nd	6,0±0,71	8,0 ±0,71	7,0 ±0,71	nd	6,0 ±0,71	7,0 ±0,71
	K	nd	8,0 ±0,71	6,0 ±0,71	10,0 ±0,71	nd	6,0± 0,71	8,0 ±0,71

nd - not detected, k - control sample without oils / nd - nie wykryto, k – próba kontrolna, bez olejków

Source: Own work / Źródło: opracowanie własne

In the case of the tested strains of pathogenic bacteria it has been observed similar phenomenon as in the case of LAB - sensitivity to the plant’s oil presence in the medium was depended on the strain and is individual. Out of the three tested strains of *Salmonella*, *S. virchov* characterized by high resistance to all oils, whereas strains of *S.typhimurium* and *S. Saintpaul* characterized by high sensitivity to most oils. It indicates that even bacteria belonging to the same genus have a varied resistance to the same substance. *E. coli* strain had a very high sensitivity to all tested oils - thyme, sage and peppermint.

The tested pathogenic strains belonging to the family *Enterobacteriaceae* are Gram-negative, with exception of *Listeria monocytogenes*. Numerous papers demonstrate that the Gram-negative strains generally exhibit a higher resistance to the essential oils than Gram-positive strains [6, 7, 14]). The obtained results do not support this regularity – it was not proved that the LAB strains were more susceptible to the test plant oils than pathogenic strains.

The thyme, peppermint and sage oils can be added to some probiotic LAB strains but tested additives (oil substances) do not increase antimicrobial activity of preparations. Their function is limited to the positive impact on the organoleptic properties of preparations.

5. Conclusion

The important feature of probiotic is antagonistic activity against harmful bacteria. Our results indicate that vegetable oils may not be equally good alternatives to antibiotic growth promoters (AGPs) as probiotics strains characterized by high antimicrobial activity since some pathogenic bacteria strains are highly resistant to the presence of plant substances. LAB sensitivity to the presence of certain essential oils is a property closely related to strains and the possibility of combining the different strains of bacteria and oils has to be always investigated case by case. There was no positive impact of the addition of essential oils on antimicrobial activity LAB. The results show that the benefits of small addition of vegetable oils to probiotic preparations are limited to increase their taste attractiveness for farm animals.

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