



***In vitro* antibacterial effects of single or combined plant extracts**

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Abstract

Single or combined extracts of black thyme, fennel, sage, wild tea and wild mint were used to evaluate *in vitro* antibacterial activity against common pathogenic and lactic acid bacteria. The combined plant extracts (1 to 1 mixing ratio) provided an entire antibacterial effect against pathogenic bacteria compared to the single plant extracts. The inhibition of lactic acid bacteria was weaker by single plant extracts than the combined plant extract. Of the plant extracts, black thyme had the strongest antibacterial activity, and this was followed by the sage, wild tea and wild mint in a descending order. The combined plant extracts with strong inhibition against pathogenic bacteria also exhibited strong inhibition against lactic acid bacteria. Therefore, the combined plant extracts with moderate inhibitory effects against both pathogenic and lactic acid bacteria could be sufficiently optimal when considering a natural feed additive to improve animal's gut health.

Key words: Antibacterial activity, herbs, extract combination, black thyme, fennel, sage, wild tea, wild mint.

Introduction

Animal feed is an important part of food chain. While nutritional value is very important to the economy of feeds and the profitability of livestock farming, increasing consumer demand has made the feed safety an essential condition for the continuity of feed suppliers and livestock farmers. Especially in recent years, dramatic food safety crises have raised public awareness of animal feed as a potential source of hazards to public health; indeed, these developments have resulted in decreased confidence in the safety of food from animal origin. In addition, one needs to realize that feed safety is not the only element that determines the safety of food of animal origin, but that the use of other products, such as medicines and natural growth promoters also plays a decisive role. Although nutritional and emotional qualities are important points for a common policy in the industry, the main concern is feed safety as part of food safety¹¹.

Of the wide range of the hazardous constituents, a group of feed antibiotics used as growth promoter in non-ruminant farm animals (poultry and pigs) has been recently banned from the animal feeds. Therefore, researches must specifically be targeted on the safe and sustainable developments of feed additives to be replaced for feed antibiotics used as growth promoter²⁰.

Herbs and spices have always helped to overcome the matters of food safety. In modern animal feeding, they are often forgotten because of the use of antimicrobial growth promoters as feed additive. These plants, their extracts and/or essential oils have long been used as remedies for some illnesses and food preservative since the ancient times. Therefore, their uses are of great importance with respect to the public health.

It has long been known that many plants rich in aroma and essential oils are widely found as wild in the Mediterranean region. Highly heterogeneous soil and climatic conditions of the region have resulted in an increased diversity of medicinal and aromatic

plants¹¹. A great proportion of the plants residing in the area have developed their secondary metabolism towards the production of essential oils, ascribing aromatic properties to the plants⁵. Most of the plants of this kind are from the family of Umbelliferae and Labiatae. Turkey is regarded as gene-centre for the latter family, representing 45 genera, 546 species and 730 taxa in Turkey⁴.

Antibacterial effects of various plants including tyme, oregano, wild tea, wild mint, sage and sweet fennel, and their derivatives were extensively investigated against food spoilage and pathogenic bacteria *in vitro* by many researchers^{2, 6, 8, 9, 12-14, 16-18}.

Medicinal and aromatic plants must be studied to bring about their *in vivo* antibacterial effects for non-ruminant animals, particularly poultry. Furthermore, food studies have only tested various doses of individual plants and/or their derivatives. Thus, a number of plant combinations and/or their derivatives were never investigated before. Therefore, we now initiated a series of experiments to test the antibacterial effects of a single and/or combination of various extracts by both *in vitro* and *in vivo* tests. Having obtained the best combination of plant extracts with a strong *in vitro* and *in vivo* antibacterial effect, then we will be able to determine a new safe and sustainable feed additives. Here we only present the results of *in vitro* experiment testing the effects of black thyme, fennel (sweet), sage, wild tea and wild mint which have been highly preferred to be consumed by the public and available for commercial use.

Materials and Methods

Plant samples: The plants used in this study are given in Table 1. Fennel (sweet), sage, black thyme, wild tea and wild mint were collected from Kahramanmaras and Gaziantep in Turkey. The plants were identified by Dr. A. Ilcim. The plants were deposited at the Department of Biology, Faculty of Science and Education, University of Sutcu Imam, Kahramanmaras in Turkey.

Table 1. Plants used in the experiment.

Plant name	Botanical name	Family	Part used
Fennel (sweet)	<i>Foeniculum vulgare</i> Mill.	Umbelliferae	Fruits
Sage	<i>Salvia pilifera</i> Montbret Aucher ex Benthams	Labiatae	Leaves
Thyme black	<i>Thymbra spicata</i> L.	Labiatae	Leaves + flowers
Wild tea	<i>Stachys pumilia</i> Banks & Sol.	Labiatae	Leaves
Wild mint	<i>Micromeria fruticosa</i> L.	Labiatae	Leaves

Preparation of plant extracts: A 20 g of each spice was ground in an ammixer and extracted for 24 h in a Soxhlet extractor with 200 mL acetone (Merck-Darmstadt, Germany) at 70°C. The crude extracts were pooled and concentrated in a rotary evaporator (Büchi Rotavapor-RE 111, 9320 Flawil, Switzerland), and then kept in small (10 ml) sterile bottles under refrigerated conditions until use^{13, 15}.

Bacteria strains: In the study, eighteen bacteria were used as test microorganisms. The bacteria, their growth temperature and used media were shown in Table 2. The spoilage and pathogenic bacteria were provided by Dr. M. Digrak (Department of Biology, Sutcu Imam University, Kahramanmaras-Turkey). The lactic acid bacteria (LAB) were produced in the present laboratory.

Determination of antibacterial effects by agar diffusion method: All test bacteria in nutrient or MRS broths (Merck-Darmstadt, Germany) were enumerated by using serial dilution method. Final cell concentrations were 10⁶-10⁷ cfu/mL. 250 µL of the bacterial suspensions was seeded on 25 mL of nutrient or MRS agars at 43-45°C. The prepared bacterial cultures were poured into Petri plates (9 cm diameter), and then agars were allowed to solidify. The agar diffusion method was used to detect the antibacterial activity of the extracts. The wells at 4 mm diameter were cut in nutrient or MRS agars. Fifty µL of 1:5, 1:10 and 1:20 w/v (weight/volume) concentrations of each extract in absolute methanol (Merck-Darmstadt, Germany) were added in the wells on nutrient or MRS agars in Trial 1. In Trial 2, only 1:5 w/v extract concentrations of study plants were mixed to provide 2 (mixing

ratio 1:1), 3 (mixing ratio 1:1:1) and 4 extract combinations (mixing ratio 1:1:1:1) at equal mixing ratio. Then, 50 µL from the combined extracts were added in the wells on agars. The absolute methanol was also used as control. The plates were incubated at suitable temperature for 18-24 h³. The diameter (mm) of inhibition zones of the extracts was measured by compass. All tests were applied as triplicate.

Statistical analysis: The data was analysed using General Linear Model (GLM). The differences between the means of groups were separated by Duncan's Multiple Comparison Test. The results were presented as the means with SEM (standard error of the mean). Significance level for the separation of the group means was set at P<0.05. Statistical analyses were carried out using the statistical program of SPSS for Windows (version 10.0).

Results

Table 3 was included with the main effects of plant extracts and various application rates on the inhibition of pathogenic and lactic acid bacteria. Fennel (sweet) was shown to have no inhibitory effect against both pathogenic and lactic acid bacteria. Among the plant extracts, the strongest inhibitory effect against all pathogenic bacteria was obtained from the extract of thyme (P<0.001), the effect of sage was moderate and that of wild tea and wild mint slight. On the other hand, there were no strong inhibitory effects of all tested plant extracts on lactic acid producing bacteria: the thyme extract with moderate inhibitory effect whereas the remaining with slight and no inhibitory effect. The results of Table 3 clearly indicated that significantly (P<0.001) high antibacterial activity against all tested bacteria was obtained from the 1 to 5 w/v extract concentrations, and that reducing the extract concentration to the 1 to 10 w/v concentration produced only a slight antibacterial activity. Furthermore, no inhibitory effects were seen with the lowest extract concentration (1/20, w/v).

The interaction effect of the plant extracts by the application concentrations are presented in Table 4. Decreasing the

Table 2. The test bacteria, their growth temperature and used media.

Test bacteria	No	Growth temperature	Used media
Spoilage and pathogenic bacteria			
<i>Enterobacter aerogenes</i> CCM 2531	P1	37 °C	Nutrient broth and agar
<i>Escherichia coli</i> DM	P2	37 °C	Nutrient broth and agar
<i>E. coli</i> O157:H7 KUEN 1461	P3	37 °C	Nutrient broth and agar
<i>Enterococcus faecalis</i> ATCC 15753	P4	37 °C	Nutrient broth and agar
<i>Klebsiella pneumoniae</i> FMC 5	P5	37 °C	Nutrient broth and agar
<i>Mycobacterium smegmatis</i> RUT	P6	37 °C	Nutrient broth and agar
<i>Salmonella enteritidis</i>	P7	37 °C	Nutrient broth and agar
<i>Salmonella typhimurium</i>	P8	37 °C	Nutrient broth and agar
<i>Staphylococcus aureus</i> Cowan 1	P9	37 °C	Nutrient broth and agar
<i>Yersinia enterocolitica</i> EU	P10	25 °C	Nutrient broth and agar
<i>Listeria monocytogenes</i> Scott A	P11	37 °C	Nutrient broth and agar
Lactic acid bacteria			
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> A42	L1	37 °C	MRS broth and agar
<i>Lb. casei</i> ssp. <i>casei</i> K64	L2	37 °C	MRS broth and agar
<i>Lb. paracasei</i> ssp. <i>paracasei</i> A27	L3	42 °C	MRS broth and agar
<i>Leu. gelidum</i> E26	L4	25 °C	MRS broth and agar
<i>Leu. pseudomesenteroides</i> E83	L5	25 °C	MRS broth and agar
<i>Weissella paramesenteroides</i> E95	L6	25 °C	MRS broth and agar
<i>Streptococcus salivarius</i> ssp. <i>thermophilus</i> S51	L7	42 °C	MRS broth and agar

concentration of extract for each of the tested plants had a significantly ($P<0.01$) lowered inhibitory effect on all test bacteria. It was also apparent that the inhibitory effects of all thyme concentrations were significantly ($P<0.01$) higher than that of the other plants.

The test bacteria which were found to be the most sensitive were evaluated if the inhibition zone is over 16 mm (Tables 3 and 4). Thus, the most sensitive bacteria amongst the pathogenic bacteria against the tested plant extracts were *Staphylococcus aureus* Cowan 1, *Enterobacter aerogenes* CCM 2531, *Mycobacterium smegmatis* RUT and *Listeria*

monocytogenes Scott A. In detail, *Staphylococcus aureus* Cowan 1 was the most sensitive bacteria against all tested extracts. *Enterobacter aerogenes* CCM 2531 and *Mycobacterium smegmatis* RUT were the most sensitive against the extracts of thyme and sage only. *Listeria monocytogenes* Scott A was the most sensitive against the extract of thyme only.

All the lactic acid bacteria except for *Lb. casei* ssp. *casei* K64 and *Lb. paracasei* ssp. *paracasei* A27 were inhibited by thyme extract (around 16 mm inhibition zone). The effect of sage extract was slight whereas there were no effects of wild tea and wild mint.

Table 3. Main effects of individual plant extracts and various application concentrations on the inhibition zone against spoilage + pathogenic bacteria (P1 to P11), and lactic acid bacteria (L1 to L7).

Bacteria no	Inhibition zone (diameter in mm) against bacteria ¹							
	Plant extracts					Application rates*, %		
	Thyme	Sage	Wild tea	Wild mint	SEM	1/5	1/10	SEM
P1	22.8 ^a	17.5 ^b	13.5 ^c	13.5 ^c	0.3	22.0 ^a	13.6 ^c	0.3
P2	18.5 ^a	13.0 ^b	9.5 ^c	9.5 ^c	0.4	15.6 ^a	11.1 ^b	0.3
P3	16.7 ^a	11.8 ^b	6.8 ^c	7.0 ^c	0.4	17.3 ^a	5.8 ^b	0.3
P4	15.7 ^a	12.0 ^b	8.5 ^c	9.0 ^c	0.3	15.0 ^a	9.5 ^b	0.2
P5	19.7 ^a	14.3 ^b	11.3 ^c	10.8 ^c	0.3	17.9 ^a	11.9 ^b	0.3
P6	21.8 ^a	16.3 ^b	13.0 ^c	13.0 ^c	0.4	20.9 ^a	12.9 ^b	0.3
P7	17.5 ^a	13.8 ^b	10.8 ^c	10.5 ^c	0.4	18.0 ^a	9.9 ^b	0.4
P8	17.8 ^a	12.8 ^b	6.5 ^d	10.3 ^c	0.4	17.6 ^a	7.8 ^b	0.3
P9	24.5 ^a	20.5 ^b	18.0 ^c	17.5 ^c	0.5	25.4 ^a	16.6 ^b	0.4
P10	13.7 ^a	6.3 ^b	4.8 ^c	5.0 ^c	0.3	13.3 ^a	3.1 ^b	0.2
P11	19.5 ^a	13.0 ^b	6.8 ^d	10.5 ^c	0.4	17.4 ^a	8.8 ^b	0.4
L1	16.7 ^a	12.8 ^b	7.0 ^d	10.3 ^c	0.4	17.9 ^a	7.5 ^b	0.3
L2	13.7 ^a	6.0 ^b	5.0 ^{bc}	4.5 ^c	0.4	12.9 ^a	3.4 ^b	0.3
L3	14.7 ^a	10.5 ^b	5.5 ^c	5.8 ^c	0.3	14.5 ^a	5.4 ^b	0.3
L4	16.8 ^a	12.0 ^b	6.8 ^c	6.5 ^c	0.3	16.9 ^a	5.8 ^b	0.3
L5	16.0 ^a	11.8 ^b	7.0 ^c	6.8 ^c	0.4	17.0 ^a	5.3 ^b	0.4
L6	16.0 ^a	12.0 ^b	6.3 ^d	10.5 ^c	0.3	16.9 ^a	6.9 ^b	0.3
L7	17.0 ^a	15.0 ^b	11.5 ^d	12.5 ^c	0.2	19.8 ^a	10.1 ^b	0.2

¹-no bacteria inhibition zone obtained; 5-9 mm: weak antibacterial activity; 10-15 mm: slight antibacterial activity; 16-20 mm: moderate antibacterial activity; and >20 mm: strong antibacterial activity. *Due to the limited number of observation, the mean values of 1/20 application rate were excluded from the mean comparison test (for details see Table 4).
^{a,b,c,d} Different letters indicates significant differences between the means of column factors (either extracts or application rates) in each of row parameters. Significant level was set at $P<0.05$.

Table 4. Interaction effects of single plant extracts by application rates on inhibition zone against spoilage + pathogenic (P1 to P11), and lactic acid bacteria (L1 to L7).

	Inhibition zone (diameter in mm) against bacteria ¹												
	Thyme			Sage			Wild tea			Wild mint			SEM
	1/5	1/10	1/20	1/5	1/10	1/20	1/5	1/10	1/20	1/5	1/10	1/20	
P1	30.5 ^a	23.0 ^b	15.0 ^d	21.5 ^b	13.5 ^d	7.5 ^e	17.5 ^c	9.5 ^e	-	18.5 ^c	8.5 ^c	-	0.6
P2	24.5 ^a	18.5 ^b	12.5 ^d	15.5 ^c	10.5 ^d	-	11.5 ^d	7.5 ^e	-	11.0 ^d	8.0 ^c	-	0.6
P3	25.5 ^a	15.5 ^b	9.0 ^e	16.0 ^b	7.5 ^c	-	13.5	-	-	14.0 ^b	-	-	0.7
P4	23.5 ^a	15.5 ^b	8.0 ^{ef}	14.5 ^b	9.5 ^{de}	-	10.5 ^{ed}	6.5 ^f	-	11.5 ^c	6.5 ^f	-	0.5
P5	27.0 ^a	19.5 ^b	12.5 ^{de}	17.0 ^c	11.5 ^e	-	14.0 ^d	8.5 ^f	-	13.5 ^d	8.0 ^f	-	0.5
P6	29.5 ^a	21.0 ^b	15.0 ^d	20.0 ^b	12.5 ^e	7.0 ^e	16.5 ^{ed}	9.5 ^f	-	17.5 ^c	8.5 ^{fg}	-	0.6
P7	24.0 ^a	17.5 ^b	11.0 ^d	18.5 ^b	9.0 ^{de}	-	15.0 ^c	6.5 ^e	-	14.5 ^c	6.5 ^e	-	0.8
P8	25.5 ^a	17.0 ^b	11.0 ^d	18.0 ^b	7.5 ^e	-	13.0 ^{ed}	0.0	-	14.0 ^c	6.5 ^e	-	0.6
P9	32.5 ^a	23.5 ^{bc}	17.5 ^d	26.0 ^b	15.0 ^{de}	8.5	22.0 ^c	14.0 ^e	6.0 ^f	21.0 ^c	14.0 ^c	6.5 ^f	0.8
P10	21.0 ^a	12.5 ^b	7.5 ^d	12.5 ^b	0.0	-	9.5 ^c	-	-	10.0 ^c	-	-	0.5
P11	25.0 ^a	19.0 ^b	14.5 ^c	17.0 ^b	9.0 ^d	6.5	13.5 ^c	-	-	14.0 ^c	7.0 ^d	-	0.7
L1	25.5 ^a	16.0 ^{bc}	8.5 ^d	18.0 ^b	7.5 ^d	-	14.0 ^c	-	-	14.0 ^c	6.5 ^d	-	0.7
L2	20.5 ^a	13.5 ^b	7.0 ^d	12.0 ^b	0.0	-	10.0 ^c	-	-	9.0 ^c	-	-	0.6
L3	21.0 ^a	15.0 ^b	8.0 ^d	14.5 ^b	6.5 ^d	-	11.0 ^c	-	-	11.5 ^c	-	-	0.6
L4	24.5 ^a	15.5 ^b	10.5 ^d	16.5 ^b	7.5 ^c	-	13.5 ^c	-	-	13.0 ^c	-	-	0.5
L5	24.0 ^a	14.0 ^c	10.0 ^d	16.5 ^b	7.0 ^c	-	14.0 ^c	-	-	13.5 ^c	-	-	0.7
L6	23.0 ^a	14.5 ^c	10.5 ^d	17.5 ^b	6.5 ^c	-	12.5 ^c	-	-	14.5 ^c	6.5 ^c	-	0.6
L7	25.0 ^a	16.5 ^c	9.5 ^d	20.5 ^b	9.5 ^d	-	16.5 ^c	6.5 ^c	-	17.0 ^c	8.0 ^f	-	0.4

¹-no bacteria inhibition zone obtained; 5-9 mm: weak antibacterial activity; 10-15 mm: slight antibacterial activity; 16-20 mm: moderate antibacterial activity; and >20 mm: strong antibacterial activity.
^{a,b,c,d} Different letters indicates significant differences between the means of column factors in each of row parameters. Significant level was set at $P<0.05$.

Table 5. Combined effects of plant extracts on inhibition zone against spoilage + pathogenic bacteria, and lactic acid bacteria.

No	Inhibition zone (diameter in mm) against bacteria ¹										
	T:S ²	T:WT	T:WM	S:WT	S:WM	WT:WM	T:S:WT	T:WT:WM	S:WT:WM	T:S:WT:WM	SEM
P1	29.5 ^a	26 ^{bc}	31 ^a	18 ^c	18.5 ^{de}	17 ^c	28.5 ^{ab}	29 ^a	21 ^d	24 ^c	1.2
P2	26.5 ^a	22.5 ^b	27 ^a	12.5 ^d	18 ^c	12.5 ^d	21.5 ^b	26.5 ^a	17.5 ^c	17.5 ^c	1.2
P3	27 ^a	20 ^c	24 ^b	12 ^e	18 ^{cd}	14 ^e	23 ^b	24 ^b	17 ^d	18.5 ^{cd}	1.1
P4	24 ^b	19.5 ^c	27 ^a	10.5 ^e	15.5 ^d	10.5 ^e	19.5 ^c	24 ^b	15.5 ^d	15.5 ^d	1.2
P5	27.5 ^a	25 ^b	28.5 ^a	14.5 ^d	18 ^c	14 ^d	23 ^b	27.5 ^a	17 ^c	18.5 ^c	1.2
P6	30.5 ^a	26 ^b	30 ^a	16.5 ^e	21 ^c	17 ^{de}	26.5 ^b	29 ^a	19 ^{cd}	21 ^c	1.2
P7	27 ^a	20.5 ^b	25 ^a	12.5 ^d	19.5 ^{bc}	14.5 ^d	21.5 ^b	25 ^a	17.5 ^c	18 ^c	1.0
P8	26 ^b	22 ^c	29.5 ^a	11.5 ^f	18.5 ^{de}	13 ^f	22.5 ^c	26 ^b	19 ^d	16.5 ^e	1.3
P9	34.5 ^a	27.5 ^d	32 ^b	19.5 ^g	25.5 ^{de}	22.5 ^f	29.5 ^c	31 ^{bc}	25 ^e	25.5 ^{de}	1.0
P10	21.5 ^a	16 ^{bc}	22.5 ^a	7.5 ^f	14 ^{cd}	11 ^e	16.5 ^b	20.5 ^a	12 ^e	13.5 ^{de}	1.1
P11	26.5 ^a	21.5 ^b	25 ^a	13 ^d	18 ^c	14.5 ^d	21.5 ^b	25.5 ^a	17 ^c	17.5 ^c	1.0
L1	24.5 ^a	22 ^b	26 ^a	13 ^c	21 ^{bc}	13.5 ^c	22 ^b	24.5 ^a	19.5 ^{cd}	18.5 ^d	1.0
L2	22 ^b	17.5 ^c	25 ^a	6.5 ^g	15.5 ^d	10.5 ^f	18 ^c	22 ^b	13 ^e	14.5 ^d	1.2
L3	23.5 ^a	18 ^{de}	21.5 ^{ab}	9.5 ^g	11.5 ^{fg}	11 ^{fg}	18.5 ^{cd}	20.5 ^{bc}	12.5 ^f	15 ^e	1.1
L4	26.5 ^a	19.5 ^c	23 ^b	12.5 ^e	15 ^d	13 ^{de}	21 ^{bc}	25.5 ^a	15 ^d	17.5 ^c	1.1
L5	24.5 ^a	17.5	25.5 ^a	13 ^c	17 ^{bc}	15 ^c	19 ^b	24.5 ^a	16.5 ^{bc}	17.5 ^{bc}	1.0
L6	25 ^a	18.5	22 ^{bc}	11 ^g	18.5 ^{de}	13.5 ^f	20 ^{cd}	23.5 ^{ab}	17 ^e	17 ^e	1.0
L7	26.5 ^a	19.5 ^d	25.5 ^{ab}	16 ^c	23.5 ^{bc}	16	21.5 ^{cd}	25 ^{ab}	21.5 ^{cd}	19 ^d	0.8

¹-no bacteria inhibition zone obtained; 5-9 mm: weak antibacterial activity; 10-15 mm: slight antibacterial activity; 16-20 mm: moderate antibacterial activity; and >20 mm: strong antibacterial activity.

²T:S, thyme and sage; T:WT, thyme and wild tea; T:WM, thyme and wild mint; S:WT, sage and wild tea; S:WM, sage and wild mint; WT:WM, wild tea and wild mint; T:S:WT, thyme and sage and wild tea; T:WT:WM, thyme and wild tea and wild mint; S:WT:WM, sage and wild tea and wild mint; T:S:WT:WM, thyme and sage and wild tea and wild mint. All the combinations of extracts were prepared from the application rate at 0.5%. ^{a,b,c,d} Different letters indicates significant differences between the means of column factors in each of row parameters. Significant level was set at P<0.05.

More importantly, these results showed that any combinations between the extract of thyme with strong inhibitory effect on pathogenic bacteria and the extracts of wild tea and/or wild mint with slight or no inhibitory effect on lactic acid bacteria would possibly exhibit an optimal antibacterial activity. All the possible combinations were, therefore, studied and the results are presented in Table 5. Highest inhibitory effects on spoilage and pathogenic bacteria were namely obtained from the extract combinations of thyme-sage (T:S), thyme-wild mint (T:WM), thyme-wild tea-wild mint (T:WT:WM), thyme-sage-wild tea (T:S:WT) and thyme-wild tea (T:WT), compared to other extract combinations. However, these combinations also produced a strong inhibitory effect against lactic acid bacteria. In contrast, combinations such as sage-wild tea (S:WT) and wild tea-wild mint (WT:WM) that produced a slight and/or moderate inhibitory effect against lactic acid bacteria were not unfortunately seen to result in a strong inhibitory effect against pathogenic bacteria. Of the extract combinations, a moderate inhibitory effect against both pathogenic and lactic acid bacteria were obtained from the extract combinations of sage-wild mint (S:WM), sage-wild wild mint (S:WT:WM) and thyme-sage-wild tea-wild mint (T:S:WT:WM).

Overall, two-way extract combinations with thyme extract exhibited antibacterial activity stronger than any other 2-way combinations without thyme extract whereas the effect of thyme extract became moderate in three- and four-way extract combinations. But still these combinations with thyme (Table 4) had stronger antibacterial activity than the single extract of thyme (Table 3). Unlikely to the effects of single plant extracts, almost all the pathogenic bacteria were significantly sensitive against all tested extract combinations.

Discussion

Fennel extract was seen to be almost inactive against both pathogenic and lactic acid bacteria. In the literature, no inhibition

on the growth of bacteria was reported with the fennel extract¹⁷ and with the fennel hydrosols¹⁸. However, Ozcan¹³ previously reported that the fennel extract had only a little antifungal effect on the growth of *Aspergillus flavus*.

The thyme extract was the most active with antibacterial activity; this was followed by sage, wild tea and wild mint in a descending order of the antibacterial activity. Numerous results have been ascribed to the antibacterial effects of thyme and sage^{1, 6, 9, 10, 12, 13, 16, 18}. From the above observations, the essential oils of these plants had stronger antibacterial effect than the extracts against both pathogenic and lactic acid bacteria. This could be of use in food industry for the safer food product in respect to consumer health, but the stronger antibacterial effect against the lactic acid bacteria cannot be preferable for the animal gut health.

It was previously determined that the extract of black thyme among tested seven plant extracts had the strongest antibacterial effect, and there were also strong antibacterial effects of sage and myrtle extracts against *E.coli* O157:H7, a food-borne pathogen, which is one of the most serious threats to feed and food safety¹⁶. The inclusion of ground leaves of black thyme at 1.0, 1.5 and 2.0% into nutrient broth was seen to inhibit the growth of *E. coli*¹⁰. Akgul and Kivanc¹⁰ also showed that various pathogenic bacteria, especially *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Salmonella typhimurium* and *Staphylococcus aureus* were strongly inhibited by the extract of black thyme. Yoshikazu et al.²¹ found that enterohemorrhagic *E. coli* O 157:H7 (EHEC) was significantly inhibited by various plant extracts. The mode of action of plant extracts was directly attributed to the inhibition against verotoxin production, not directly the bactericidal effect. From this point of view, one could speculate that the inactivation of pathogenic bacteria within the living gut microflora only by the means of inhibiting toxin production still have higher potentially for the health of animals when medicinal and aromatic plants and their derivatives are considered since they could maintain the whole gut

integrity as healthier as possible in a natural balance.

In the literature, studies on the antibacterial effects of wild mint and wild tea were seen to be rare. In a study it was reported that the essential oil of wild mint was seen to inhibit *Escherichia coli* and *Enterobacter aerogenes*, *Staphylococcus aureus* and *Salmonella typhimurium*¹⁹. Digrak et al.⁷ found that the extract of wild tea inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Enterobacter aerogenes*. In contrast, no strong or moderate antibacterial effects were seen with the single extracts of wild tea and wild mint in the present study. The effects of these plant extracts on bacterial growth were, however, apparent when combining them with other plant extracts.

No studies on the effects of various combinations of plant extracts were previously conducted to determine an antibacterial activity suitable for incorporating in foods and feeds where a naturally food and/or feed additive is desired.

With the single plant extracts, *Staphylococcus aureus* Cowan 1, *Enterobacter aerogenes* CCM 2531, *Mycobacterium smegmatis* RUT and *Listeria monocytogenes* Scott A among pathogenic bacteria were strongly inactivated: the effects of thyme and sage were the strongest on these bacteria. However, with the combined plant extracts, there was a great degree of inhibition on almost all tested pathogenic bacteria compared with the single plant extracts. On the other hand, the inhibitory effects of single plant extracts (only thyme) on lactic acid bacteria were weaker than that of combined plant extracts. The combined plant extracts with stronger inhibitory effects against pathogenic bacteria, in fact, failed to exhibit a weak inhibition against lactic acid bacteria. In contrast, the extract combinations with weaker inhibitory effect against lactic acid bacteria did not provide strong antibacterial activity for pathogenic bacteria in the present case. Of the extract combinations, we can suggest that a moderate inhibitory effect against both pathogenic and lactic acid bacteria obtained from the extract combinations of sage-wild mint (S:WM), sage-wild tea-wild mint (S:WT:WM) and thyme-sage-wild tea-wild mint (T:S:WT:WM) could be considered as useful plant combinations for the production of natural feed additives.

However, further studies on determining the optimal mixing ratios between the extract combinations are needed for the best formulation to prepare a suitable feed additive. Future trials are on the way to explicit the effects of combined plant extracts on the performance and gut health of poultry in this manner.

Conclusions

The use of plant extracts could provide a potential alternative to feed antibiotics in animal nutrition. The results of present *in vitro* study indicated that appropriate combinations between the single plant extracts could evidentially provide an optimal antibacterial activity against both pathogenic and lactic acid bacteria compared to individual plant extracts when considering the use of natural feed additives in animal nutrition.

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