DANISH MEAT
RESEARCH INSTITUTE

Report
"BerryMeat"
Antimicrobial effect for different preparations from 8 plants during storage at 18°C for 1½ year.

19. marts 2014
Project 2000248-13
FH/

Flemming Hansen

Summary

Introduction

In the project, four different formulations of the 8 selected herbs & berries has been prepared (freeze-dried, oven-dried, raw blended and raw blended & pasteurized).
In this study, the antimicrobial activity has been investigated shortly after preparing the formulations and again after 1 year and 1½ year.
The formulations were added to a broth model system (8 % w/V), inoculated with *Salmonella, E. coli* and *L. monocytogenes* at approx. 1000 cfu/ml. The growth/inhibition of the 3 types of bacteria was measured at day 2 or 3 and at day 6 during incubation at 37°C.

Objective

The objective of the study was to investigate the difference in antimicrobial activity present in the four different formulations. Further to investigate whether this antimicrobial activity is lost during storage at ÷ 18°C for up to 1½ year. As two of the formulations are dried, these are rehydrated to the original water content before adding 8% of the formulation to the broth system. Thus equivalent amount of the original herb/berry was compared

Conclusion

In general, it can be concluded that the raw, finely ground/blended material seems to have the highest antimicrobial activity, independent of being pasteurized or not. If one need to use a dried product, the freeze drying process seems much better than the "oven-drying" process in order to preserve the antimicrobial compounds.

It seems fair to conclude, that storage at ÷18°C is able to preserve the antimicrobial activity for up to 1½ year, as no significant reductions in activity can be observed during storage. The small differences observed in the study , may all be attributed to the relative large sample variation and analysis uncertainty in the test system.

Materials & Methods

Principle

8 different herbs and berries, pre-treated/prepared in 4 different ways (freeze-dried, oven-dried, blended or blended and pasteurized) were analyzed for antimicrobial activity shortly after being prepared and after storage at ÷18 °C for approx. 1 year respectively 1½ year. The 8 plants were added at 8% w/v to Brain Heart Infusion supplemented with extra 2.5% NaCl. The broth was inoculated with *E. coli* (3 strains), *Salmonella* (3 strains) and *L. monocytogenes* (5 strains) at a level around 3 log cfu/ml, and incubated at 10°C for to 6 days. Shortly after inoculation, after 3 days and after 6 days, the bacterial count was determined by spread plate using two selective agars.

Bacteria used in the experiment

For determining growth inhibition, a cocktail of *L. monocytogenes* (5 strains), *Salmonella* (3 strains) and *E. coli* (3 strains) was used. All strains was supplied by the Danish Meat Research Institute Strain Collection (DMRICC)

L. monocytogenes

- DMRICC 3012 (Unknown serotype , environment, abattoir)
- DMRICC 4106 (serotype 1/2a, clinical isolate)
- DMRICC 4124 (serotype 1, meatproduct)
- DMRICC 4127 (serotype 4, meat product)
- DMRICC 4140 (serotype 1, bacon)

E. coli

- DMRICC 4233 (O111:H÷ VT neg, clinical isolate)
- DMRICC 4235 (O26:H÷ VT neg, clinical isolate)
- DMRICC 4987 (O157 VT neg, beef)

Salmonella

- DMRICC 4983 (*S.* Dublin, beef)
- DMRICC 4984 (*S*. Typhimurium DT193, cattle feces)
- DMRICC 4985 (*S*. Derby, pig feces)

The strains were maintained as frozen stock at -80°C. Initially the cultures were thawed and streaked onto BHI-A (Brain Heart Infusion agar Oxoid, CM1136) and grown overnight at 37°C. Next day, material from one colony was transferred to 10 ml BHI (Brain Heart Infusion, Oxoid CM1135), and incubated statically for 24 hours at 37 °C to a final count of approx. 10^9 cfu/ml. One ml from each of the 11 overnight cultures were mixed, and inoculated to a level at 10^3 – 10^4 cfu/g into BHI added extra 2.5% NaCl. Afterwards aliquots of 20 ml were aseptically transferred to a Blue-Cap bottle and used as growth medium for the inhibition test.

Preparation and incubation of plant material and samples

Four different formulations made from the same starting material from eight different plants (table 1) were tested.

- Freeze-dried
- Oven-dried (at 50°C)
- Finely ground/blended
- Finely ground/blended, pasteurized in 92°C hot air

Thus, in total 32 different samples were prepared for testing. All samples were stored at ÷18°C (vacuum packed) until the day of testing. Prior to testing the samples were thawed overnight in a refrigerator.
Based on the data for drying loss (table 1), the dried samples were rehydrated to the original water content, using sterile, distilled water. Afterwards 8% w/vol of the plant material was added to the inoculated growth medium, thoroughly mixed, and incubated at 10.0° ± 1.0°C with gentle agitation.
As "no-inhibition" control, samples of inoculated BHI+2.5% NaCl was included and as "inhibition" control, samples of inoculated BHI+2.5% NaCl added 0.05% purified ß-acids from hops (Humulus lupulus; BetaTec Hop Products, Nuremberg Germany) was included.

Analyses

pH measuring
Shortly after adding plant material to the sample, pH was measured (pH-meter PHM 93, Radiometer, Copenhagen).

Bacterial counts
After inoculation of the initial BHI+2.5% NaCl, but before adding plant material, three samples of 1 ml were removed to determine the inoculation level. After 2 or 3 days incubation and again after 6 days incubation, one ml was removed, in order to determine the increase in bacterial count
The 1 ml sample was 10-fold diluted in w/v 0.85 % saline + 0.1 % peptone, 100 µl of relevant dilutions was spread onto SSI Enteric Medium (Statens Serum Institut, Cat.no. 34121, SSI, Denmark) respectively Oxford agar (Oxoid CM0856+ SR206E). SSI agar was incubated at 37°C for 24 hours and *Salmonella* and *E. coli* estimated as the number of black and red colonies respectively. Oxford agar was incubated 48 hours at 37°C and *L. monocytogenes* was estimated as black colonies.
Each of the 4 preparations from the eight plants was tested in two duplicate broth samples and each broth sample was tested in duplicate.

Variables investigated during the project

In the first experiment the anti-microbial effect of the plants pre-treated/prepared in the 4 different ways was investigated, shortly after they were prepared (less than one month).
A similar experiment was carried out after storage of the preparations for approx. 12 month at ÷18°C and again after approx. 18 months storage.

Results

All growth data form the broth model experiment was transferred to Excel spreadsheet and log transformed. The growth was expressed as the difference between the measured mean log count at day 2 or 3 respectively day 6 and the initial log count. Further the total bacterial count at day 6 was evaluated and partly used to quantify the antimicrobial activity.
In some cases, a negative growth resulted, indicating that killing of the bacteria or a reduction has taken place. However, the quantification of the reduction was compromised by the use of different dilutions for the spread plate technique when performing the colony counting. Thus, any reduction observed is only reported as “no growth at day 6” (=bactericidal effect). In order to categorize the 32 different formulations in a n easy way, the following scheme was used

(3) Strong bacteriostatic effect = less that 1 log growth at day 6

(2) Intermediate bacteriostatic effect = less than 3 log growth at day 6

(1) Slight bacteriostatic effect = Growth above 3 log AND total bact. count less than 7.0 log at day 6

(0) No bacteriostatic effect = total bacterial count at day 6 above 7.0 log

Based on the above criteria the 4 formulations for each of the 8 plants were categorized as can be seen in table 2.

Conclusions

In general, it can be concluded that the raw, finely ground/blended material seems to have the highest antimicrobial activity, independent of being pasteurized or not. If one needs to use a dried product, the freeze-drying process seems much better than the “oven-drying” process in order to preserve the antimicrobial compounds.

It seems fair to conclude, that storage at ÷18°C is able to preserve the antimicrobial activity for up to 1½ year, as no significant reductions in activity can be observed during storage. The small differences observed in the study, may very well be attributed to the relative large sample variation and analysis uncertainty in the test system.

Appendix 1

Table 1: Calculated water loss for freeze-dried respectively oven-dried plant material

Plant	Water loss % (drying loss %)			
	freeze-dried	Oven-dried	Blended	Blended/past.
Aronia (black chokeberry)	**82.4**	**82.8**	**No drying loss**	**No drying loss**
Lingonberry	**86.2**	**86.6**		
Redcurrant	**82.0**	**84,0**		
Blackthorn (Sloe)	**78.5**	**76.7**		
Ramson	**68.2**	**67.2**		
Horseradish	**65.0**	**65.0**		
Summer savory	**85.4**	**86.2**		
Sage	**81.0**	**86.1**		

Table 2: pH values for broth model added 8 % of the 8 different herbs and berries (no significant difference between the 4 formulations within one plant species)

	Less than 1 month	After 1 year	After 1½ year
Aronia	6.4	6.4	6.2
Lingonberry	5.1	5.1	4.9
Redcurrant	5.1	5.1	5.1
Blackthorn (Sloe)	6.0	6.1	5.9
Ramson	6.9	7.1	6.9
Horseradish	6.9	6.9	6.8
Summer savory	7.0	7.1	7.0
Sage	7.0	7.1	7.0

Table 3a: Antimicrobial activity of four formulations from 8 plants against *L. monocytogenes* shortly after preparation (1 month), after 1 year (12 month) and after 1½ year (18 months)

Plant	Antimicrobial activity											
	Freeze-dried			Oven-dried			Blended			Blended/past.		
	1	12	18	1	12	18	1	12	18	1	12	18
Redcurrant	2	2	3	0	0	2	3	3	3	2	3	3
Lingonberry	2	2	2	1	0	2	3	3	3	2	3	3
Aronia	0	0	0	0	0	0	0	0	0	0	0	0
Blackthorn	0	0	0	0	0	0	0	0	0	0	0	0
Sage	2	3	3	3	3	3	3	3	3	2	2	3
Summer savory	1	0	0	0	0	0	1	0	0	0	0	0
Horseradish	3	3	3	3	3	3	3	3	3	3	3	3
Ramson	3	3	3	3	3	3	3	3	3	3	3	3

Table 3b: Antimicrobial activity of four formulations from 8 plants against *Salmonella* shortly after preparation (1 month), after 1 year (12 month) and after 1½ year (18 months)

Plant	Antimicrobial activity											
	Freeze-dried			Oven-dried			Blended			Blended/past.		
	1	12	18	1	12	18	1	12	18	1	12	18
Redcurrant	3	2	3	0	0	2	3	2	3	3	3	3
Lingonberry	3	2	3	1	0	3	3	3	3	3	3	3
Aronia	0	0	0	0	0	0	0	0	0	0	0	0
Blackthorn	0	0	0	0	0	0	0	0	0	0	0	1
Sage	0	0	0	0	0	0	0	0	0	0	0	0
Summer savory	0	0	0	1	1	1	0	0	0	0	0	0
Horseradish	2	1	2	2	1	2	3	2	2	3	3	3
Ramson	3	3	3	3	3	3	3	3	3	3	3	3

Table 3c: Antimicrobial activity of four formulations from 8 plants against *Escherichia coli* shortly after preparation (1 month), after 1 year (12 month) and after 1½ year (18 months)

Plant	Antimicrobial activity											
	Freeze-dried			Oven-dried			Blended			Blended/past.		
	1	12	18	1	12	18	1	12	18	1	12	18
Redcurrant	3	3	3	1	1	3	3	3	3	3	3	2
Lingonberry	3	3	3	1	1	3	3	3	3	3	3	3
Aronia	0	0	0	0	0	0	0	0	0	0	0	0
Blackthorn	0	0	0	0	0	0	0	0	0	0	0	0
Sage	0	0	0	0	0	0	0	0	0	0	0	0
Summer savory	0	0	0	0	0	0	0	0	0	0	0	0
Horseradish	2	1	2	2	2	2	3	1	2	3	3	3
Ramson	3	3	3	3	3	3	3	3	3	3	3	3