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ANTIMICROBIAL PROPERTIES OF TRADITIONAL BREWING HERBS

- Ledum palustre, Myrica gale & Humulus lupulus

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ABSTRACT

In this study, three different brewing herbs that have been used through the history are evaluated as inhibitors of common beer spoilage organisms. The three species are *Ledum palustre* (Marsh Tea), *Myrica gale* (Bog Myrtle) and *Humulus lupulus* (Hops). Experimental batches of 10 L were made with all the three herbs and one without any additives. For each herb, two batches were made with different concentration, one batch with 3g/L and the other with 6g/L. All batches were treated the same and fermentation pattern for all of them were similar. Inoculations of four common beer spoilage organisms were practiced in order to examine microbial resistance of the different beers. Antibacterial activity was analyzed by membrane filtration and by measure the optical density during the incubation time. Both *Humulus lupulus* and *Myrica gale* showed clear resistance to the three gram-positive bacteria.

INTRODUCTION

Throughout history, several different plant species have been used as beer additives for flavour and above all as preservatives. Particularly two species, *Myrica gale* and *Humulus lupulus* were widely used as beer additives in Europe (Behre 1999). The antibacterial properties of beer additives were of greater importance before the industrialization of brewing and the discovery of pasteurization. Today Hops (*Humulus lupulus*) dominates the brewing, but nowadays it is mainly used for flavour and bitterness.

The Hop cones of female plants contain lupulin glands, which include both the resins and essential oils (Briggs *et al*. 1999). The resins compose two acids, α-acid and β-acid, which are responsible for its antibacterial activity and bitterness (Lewis and Young 2001), and which inhibit gram positive bacteria by causing breakdown of the trans-membrane pH gradient (Simpson 1993).

Bog Myrtle appears to been used as a beer additive earlier than hops, within Bog Myrtles natural distribution area. Archaeological finds suggests that the use of Bog Myrtle was limited to regions where it occurred naturally (Behre 1998). The finds also suggest that Bog Myrtle have been used for brewing already for over 2000 year in the area of the Rhine in the northern Netherlands (Behre 1998). The first written source about its use in brewing is by Hildegard, the learned abbes, in the 12th century. After the 12th century several other written sources about its use in brewing are available (Hofsten 1960). In the early Middle Ages there is a significant increase in the quantity of hops in archaeological finds, and during late medieval period there is strong competition between the different beers and eventually hopped beer comes to dominate (Behre 1999). Karl Behre mentions that beer brewed with Bog Myrtle was not as stable as hopped beer. Another disadvantage with Bog Myrtle beer could be rumors of its possible toxicity. In Sweden, from the 16th century and onwards hops was the main beer additive, but Bog Myrtle was still used by peasants and during hop shortages (Thunæus 1968). Information from the Nordic Museum in Stockholm also confirms the use of Bog Myrtle and shows that Bog Myrtle was used by Swedish peasants until the late 19th century (Hofsten 1960).

Bog Myrtle (*Myrica gale*) is a shrub growing on acid bogs and sandy soils in North West Europe (Behre 1998). Bog Myrtle produces a volatile oil that is stored in glands on the surface

of the leaves, flowers and fruit (Carlton et al 1992). All parts above the ground have been used as an additive in beer (Malterud 1982; Behre 1998). Karl E Malterud showed in 1982 that the fruits of Bog Myrtle have antibacterial properties and the flavonoid Myrigalon A seems to be responsible for this activity (Malterud 1982). Evalad Sandegren experiments with Bog Myrtle in a work titled *"Bog myrtle (Myrica gale) and other substitutes for hops in former times"*, and reports that experimental brews made with different parts of the plant all show some bacteriostatic effect (Hofsten 1960),

Ledum palustre has sometimes been mistaken for Bog Myrtle. These two plants both have very distinct smells, but are easily distinguished. Also, the natural distribution areas are different. The reason for the confusion about the two species seems to be their names. In Germany and Sweden both plants have been called "*Pors*" (Hofsten 1960). Marsh Tea (*Ledum palustre*) is an evergreen shrub growing plant and is found in northern Europe, northern America and northern Asia. Marsh Tea grows well in bogs and has a very strong smell that can even cause headaches. The whole plant is reported as toxic, and effects on the central nervous system and aggressive behavior has been reported (Gretšušnikova *et al.* 2010). The main constituents of the essential oil are palustrol, ledol and myrcene (Butkiene *et al*. 2008) but the composition of the oil varies significantly with habitat (Gretšušnikova *et al.* 2010). Myrcene is also found in the essential oil of Hops (Brewing 2004). According to Karl-Ernst Behre (1998) *Ledum palustre* has been used for beer flavouring but not as a preservative.

In this project, individual beers have been made using three different brewing herbs and at different concentrations. In total, seven beers were produced. One control beer without any herb and two brews for each brewing herb using different amounts. Fermentation patterns for all the brews have been monitored by regularly gravity and pH checks. After complete fermentation, all the beers were inoculated with four common beer spoilage organisms (BSO). Antimicrobial activity was measured by spectrophotometer and membrane filtration.

MATERIALS & METHODS

Production of test beers

The Seven different beers were made with wort obtained from Stallhagen Brewery (Mariehamn, Åland). The wort was frozen and stored in a freezer at -18°C and subsequently thawed in a sink of hot water before boiling. The specific gravity of the wort was measured to 42 Oechsle degrees by using an Alla hydrometer. The herbs used in the experiment were all dried. Northern Brewer hops of 2009 crop with alpha acid content of 9.3% were bought via Humlegården (Vallentuna, Sweden). The hops were dried hop cones and of German origin. This particularly hop was chosen because of it is popularity and all round use. *Myrica gale* was ordered from Saxens Örter (Edsbro, Sweden) and *Ledum palustre* was handpicked in Nåsten (Uppsala, Sweden). The *Ledum palustre* were dried at room temperature for twelve days.

Table 1. Brewing herbs used in the experiments

Common name	Latin name	Format	Origin
Hops	*Humulus lupulus*	Dried hop cones	Germany
Bog Myrtle	*Myrica gale*	Dried leaves	Sweden
Marsh Tea	*Ledum palustre*	Dried leaves	Sweden

Two brewing batches were made for every herb using different amounts of plant material, one with 3g/L (Low) and another with 6g/L (High). From here on, the two different concentrations will be referred as “Low” and “High”.

Table 2. Different concentrations of the brewing herbs

	Unhopped	*H. lupulus*		*M. gale*		*L. palustre*	
concentration	-	Low	High	Low	High	Low	high
g/L	-	3	6	3	6	3	6

Ten liters of wort were used for every batch and the wort was boiled for 20 minutes before the actual “wort boil” in order to ensure sterility. Then a 60 minutes wort boil was performed. The additions of herbs into the boil were done at different times.

Table 3. Addition times for brewing herbs

Herb	Additions			
	1st		2nd	
	Amount*	Time**	Amount*	Time**
Hops "Low"	20	60	10	5
Hops " High"	40	60	20	5
Bog Myrtle "Low"	15	20	15	5
Bog Myrtle "High"	30	20	30	5
Marsh Tea " Low"	25	60	5	5
Marsh Tea "High"	50	60	10	5

*Amount of herb added (g), **Boiling time (min)

Due to the need of isomerization of hop resins, the first additions of hops were at the beginning of the boil. The alpha-acids are badly solubilized in wort, however, during boiling a rearrangement of alpha acids to iso-alpha-acids occurs and solubility is increased (Fix 1999). The reason for the long boiling time when it comes to Marsh Tea is because of its toxicity. The whole plant is poisonous and could cause headache and aggressive behavior (Gretšušnikova *et al*. 2010). Components of the essential oils in Bog Myrtle that could have antibacterial properties were assumed to be quite volatile. Therefore none of this herb was added until the last 20 minutes. The wort was cooled after boiling by putting the pot in an ice bath for approximately 20 minutes. Due to evaporation, all batches were adjusted with distilled water to ensure the same gravity, 40 Oechsle degrees.

The cooled wort was then transferred to demijohns (dame-jeanne) through a sterile funnel. Aerating was done by shaking the demijohns vigorously. The prepared yeast cultures were then added and sterile airlocks were put on the top of the demijohns. The fermentation temperature was maintained at 21°C (±1°C). After a week the beer was transferred to the secondary fermenter by using a sterile auto siphon. The beer was left in the secondary fermenter for two weeks of further fermentation and maturation. The beers were bottled in 200 ml Duran bottles. The bottles were filled almost to the top to minimize head space and final volume was approximately 280 ml.

Yeast Preparation

The yeast strain used in this study was Safale S-04 Fermentis, purchased from Humlegården (Vallentuna, Sweden). For every batch, 10 g of dry yeast were weighed out into a sterile Erlenmeyer flask and re-hydrated with 100 ml of sterile tap water at 28°C. The yeast suspensions were then stirred for 30 minutes prior to pitching

Preparation of test organisms and inoculation of beer

All bacteria were from Culture Collecetion University of Gothenbourg (CCUG) apart from the *Lactobacillus brevis* strain, which were provided by Stefan Roos at SLU (Uppsala, Sweden). The other test organisms were *Lactobacillus buchneri* (CCUG 21532), *Pediococcus damnosus* (CCUG 32251) and *Acetobacter pasteurianus* (CCUG. The strains were all grown and maintained on MRS agar. *L. brevis*, *L. buchneri* and *P. damnosus* were grown at 30°C in anaerobic jars for 48 h. BBL Gas-Pak envelopes were used to create anaerobic conditions. *A. pasteurianus* were grown aerobically at 30°C for 96 h. They were all stored at 4°C and subcultured every second week.

The bacteria suspensions to be inoculated were prepared by dissolving fresh colonies in 0.9% saline solution. Suspensions were vortex-mixed and cell concentration was determined microscopically in a Bürker chamber and also by measuring the optical density (OD) at 600nm in a Shimadzu UU-1601 spectrophotometer. The concentration used for all the inoculations were 3 x 10^6 cells into 280 ml batches. The same amounts of bacteria for inoculation of beer were used by Fernandez and Simpson 1992.

Beer analyses

The fermentation patterns were checked daily for the first 7 days by measuring the gravity and pH. All samples were of temperature 20°C and degassed by pouring between two beakers twenty times.

The OD of inoculated samples was measured at 600 nm every second day for 10 days. At the last day, the beer was analyzed by membrane filtration. The samples that were filtered had been incubated in dark for the same period of 10 days and at the same temperature 26°C. However, these samples had not been opened during the incubation period like the samples that were measured by spectrophotometer. The samples to be analyzed by membrane filtration

had been done in duplicates. Membrane filtration was carried out using a Buchner funnel and flask connected to a vacuum line. All solutions were filtered through 0.45 μm Millipore filters with a sample size of 100 ml. After filtration, the filter was transferred to MRS+Cyclohexamide agar plates using sterile tweezers, and plates were incubated as described earlier. The addition of Cyclohexamide was used to inhibit yeast growth, 20ug/ml (Lewis and Young 2001).

RESULTS

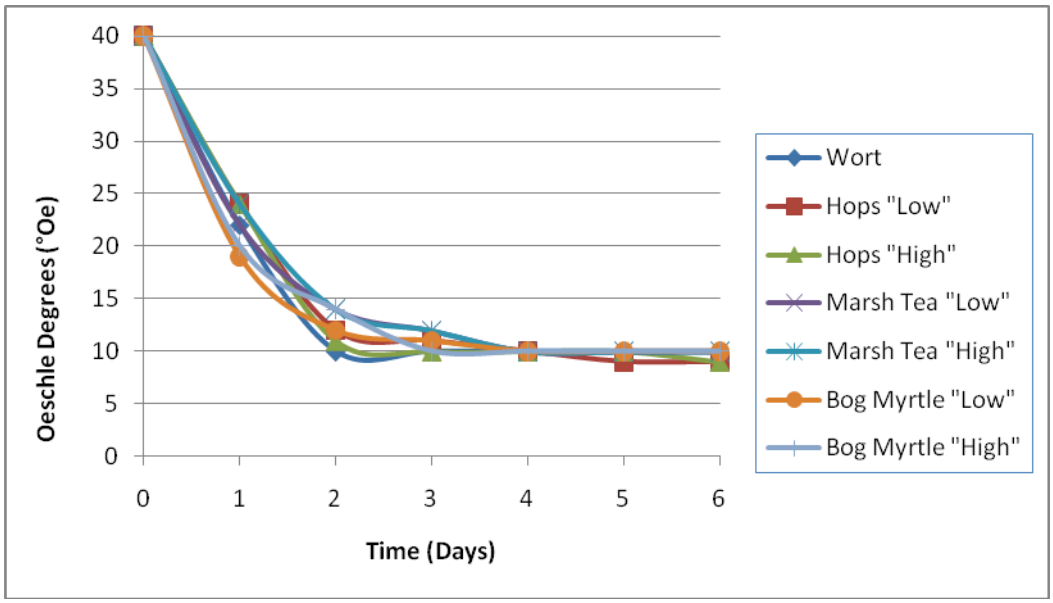

Fig. 1. Change in gravity during the first six days of fermentation

All batches had the same start gravity of 40 °Oe and dropped in gravity to 10(-1) °Oe. Data is also shown in the appendix.

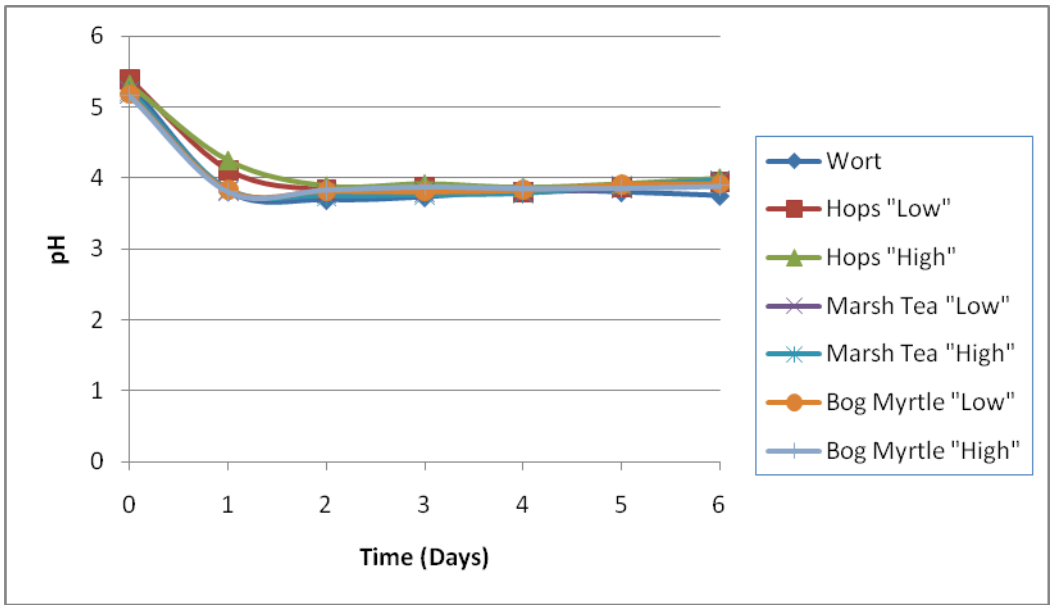

Fig. 2. Change in pH during the first six days of fermentation

The drop in pH also followed a similar pattern for all brews.

Table 4. Growth of test organisms in the different beers, analysesd by membrane filtration

Beer	Control	Organisms			
		L. brevis	*L. buchneri*	*P. damnosus*	*A. pasteurianus*
Wort	+	+	+	+	+
Hops "Low"	-	-	-	-	+
Hops "High"	-	-	-	-	+
Marsh Tea "Low"	+	+	+	+	+
Marsh Tea "High"	+	+	+	+	+
Bog Myrtle "Low"	+	+	+	+	+
Bog Myrtle "High"	-	-	-	-	+

(+) indicates growth of colonies on the membrane filter and (–) means no growth.

The membrane filtrations were done in duplicates and all showed the same results except one of the two filtrations of hopped beer with high concentration. One of the plates had four colonies of *P. damnosus*. The colonies on all the other positive plates were too many to count or too smeared/close to each other.

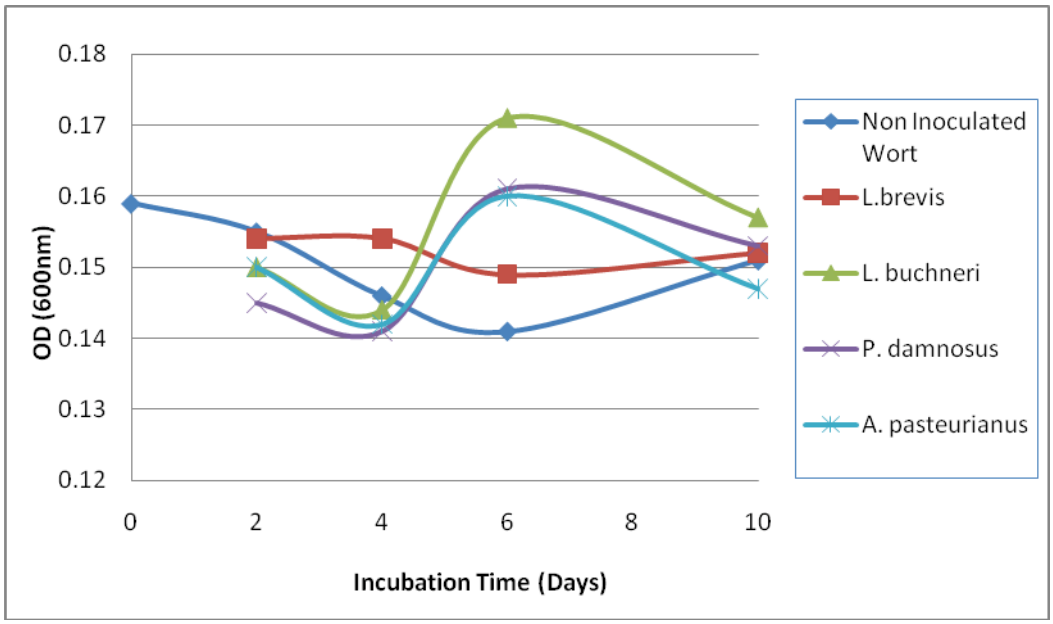

Fig. 3. Growth comparison between non inoculated wort and wort inoculated with four different beer spoilage organisms

In fig. 3, all three bacteria apart from *L.brevis* had a significant increase in growth at day 4, with a peak at day 6. The non-inoculated sample decreases in optical density until day 6, then a slight increase can be noticed.

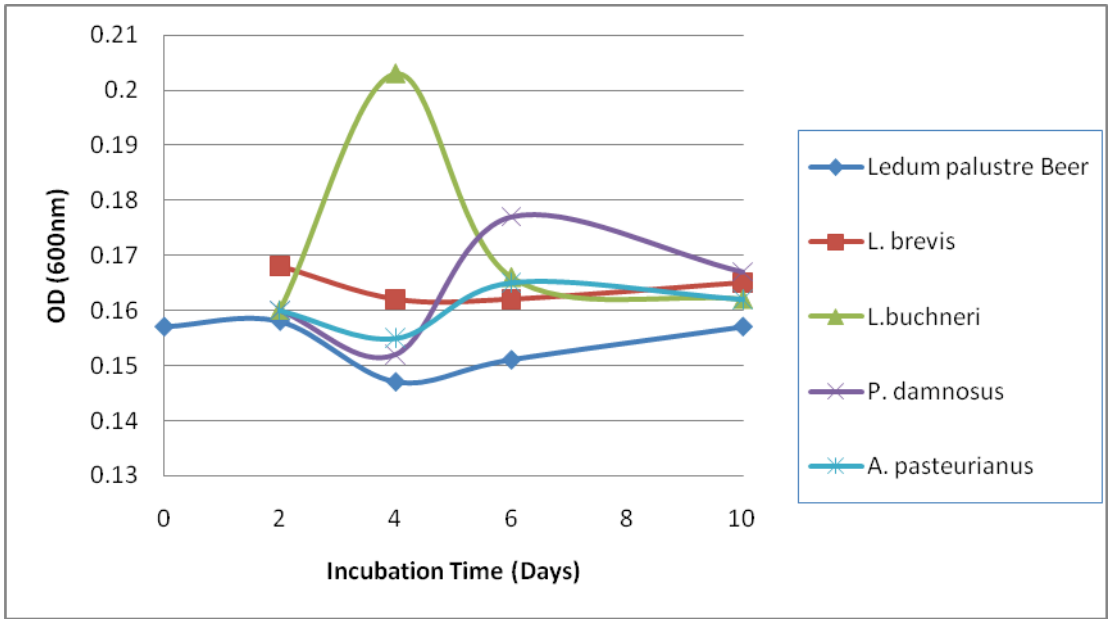

Fig. 4. Growth comparison between non inoculated *Ledum palustre* beer (Low) and samples inoculated with four different beer spoilage organisms

In the *Ledum palustre* beer (Low), all strains increase in opitical density. Although *L. brevis* seems to grow very slow and have a very small increase in growth compared to the other strains. *L. buchneri* grows particulary well in the *Ledum palustre* beer. Also the non-inoculated beer starts to increase in OD at day 4.

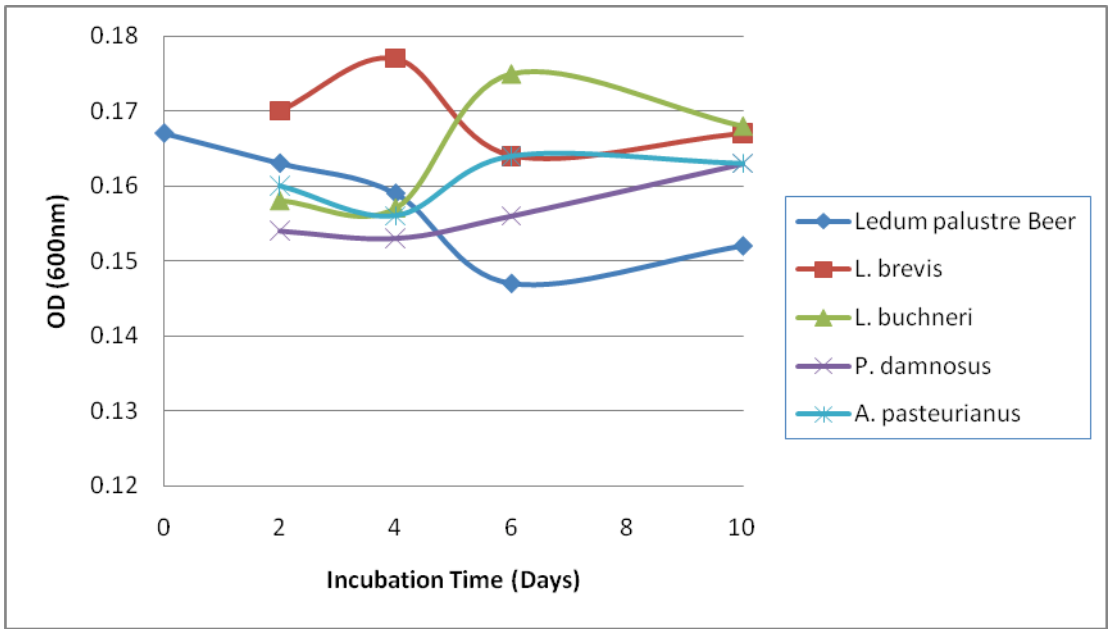

Fig. 5. Growth comparison between non inoculated *Ledum palustre* beer (High) and samples inoculated with four different beer spoilage organisms

All strains seem to grow in the *Ledum palustre* beer (High) and at day 6 the non-inoculated sample slowly increases in OD.

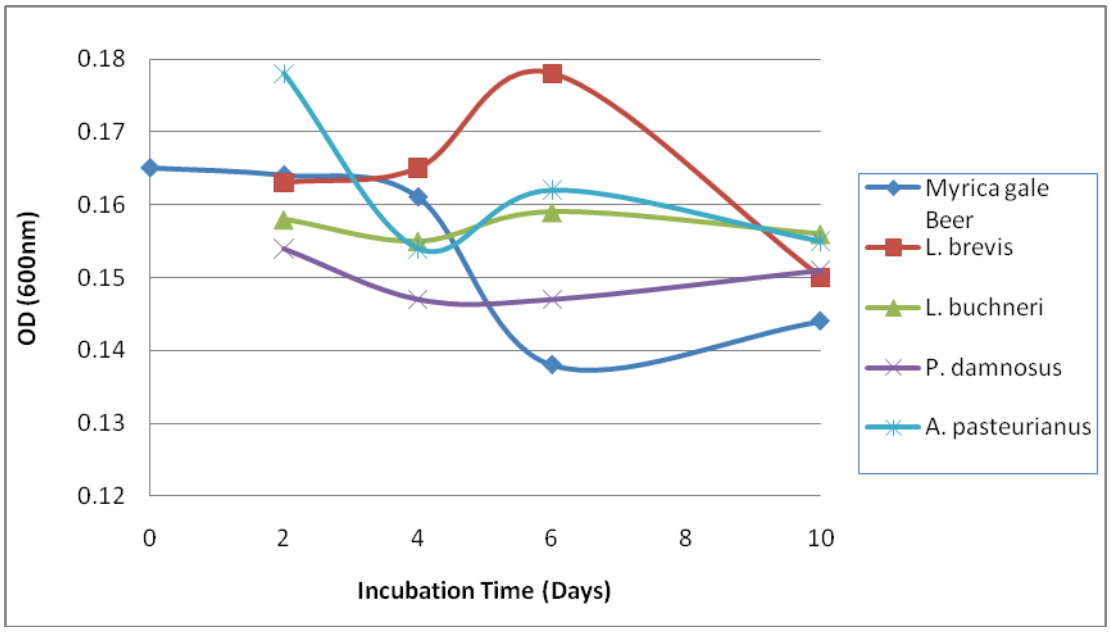

Fig. 6. Growth comparison between non inoculated *Myrica gale* beer (Low) and samples inoculated with four different beer spoilage organisms

In fig. 6, it can be seen that *L. brevis* grow better than the other bacteria. The control beer also has an increase in OD that start around day 7.

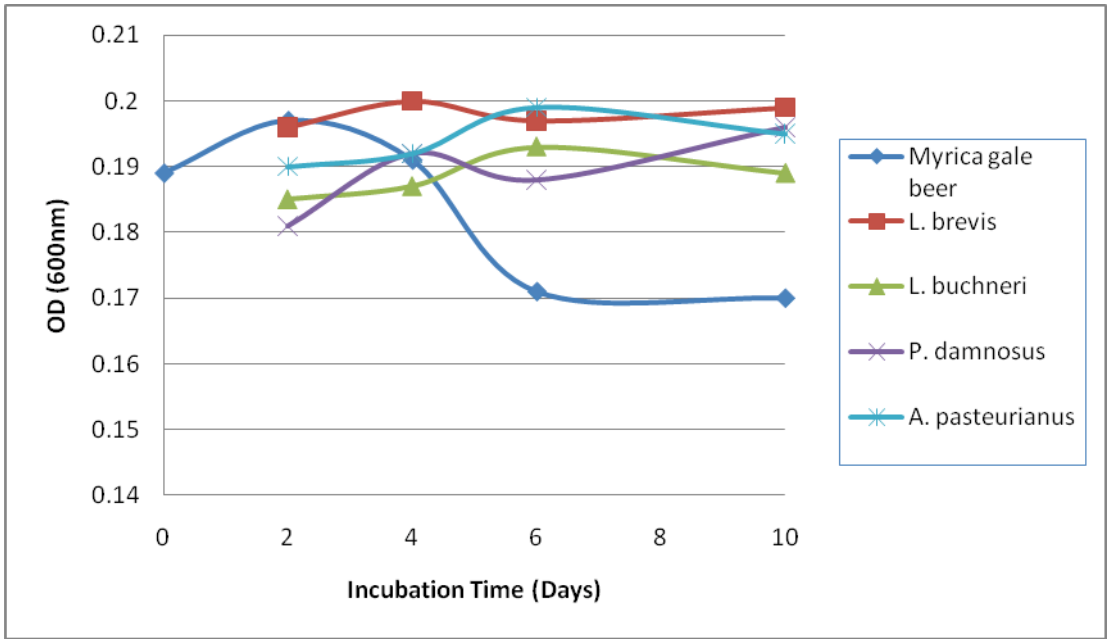

Fig. 7. Growth comparison between non inoculated *Myrica gale* beer (High) and samples inoculated with four different beer spoilage organisms

The Myrica gale beer (High) decreases in OD at day 2 and level out at day 6. The inoculated samples just have small fluctuations in OD.

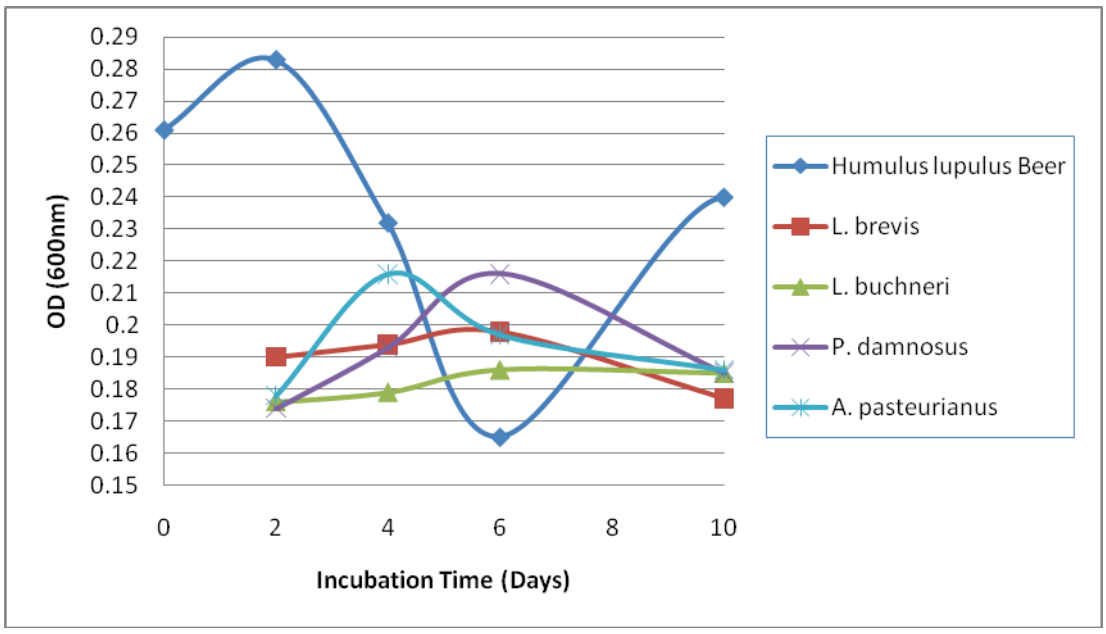

Fig. 8. Growth comparison between non inoculated *Humulus lupulus* beer (Low) and samples inoculated with four different beer spoilage organisms

In fig. 8 the *Humulus lupulus* beer (Low) shows much higher OD from the beginning compared to the other samples. There is a sharp decrease but at day 6 a rapid increase in OD takes place. *A. pasteurianus* and *P.damnosus* grow more readily than the two other bacteria.

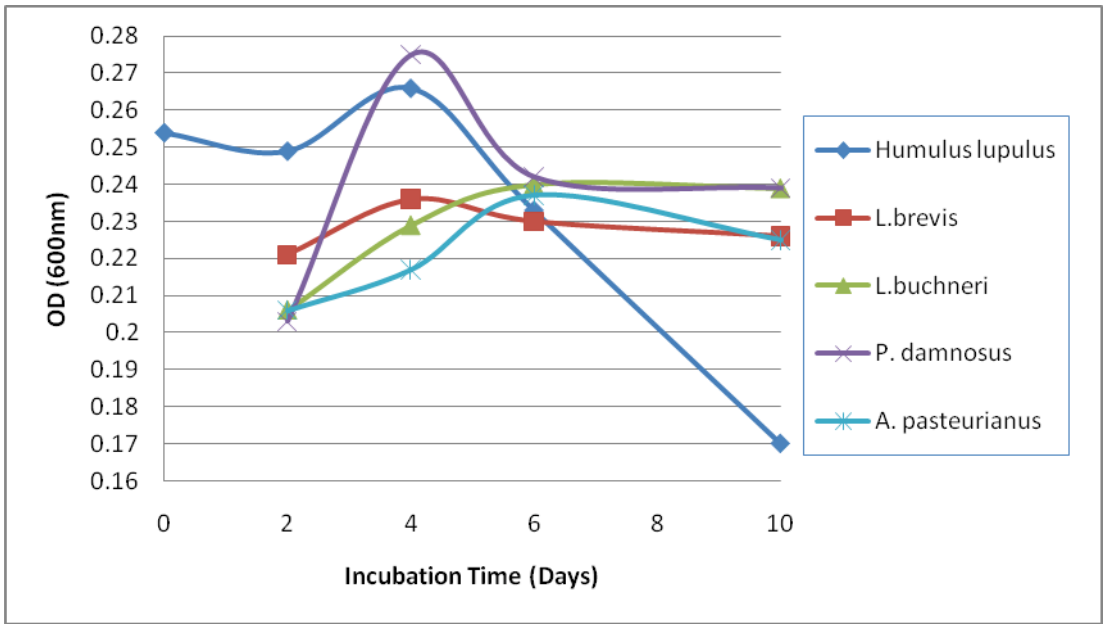

Fig. 9. Growth comparison between non inoculated *Humulus lupulus* beer (High) and samples inoculated with four different beer spoilage organisms

The non-inoculated sample of *Humulus lupulus* beer (High) decrease in OD at day 4 and no growth is observed from that day. Increase in OD can be noticed for all inoculated samples; however, only *P. damnosus* has a sharp increase in OD.

DISCUSSION

All fermentations showed almost the same fermentation pattern as far as gravity and pH is concerned. They all started quickly and after primary fermentation gravity fell to 9 (-1) degrees Oechsle (Fig. 1.). The pH differed slightly between different beers (Fig. 2.); however all were within the normal range of pH 3.8-4 except the wort (Campbell 2003). The similar patterns show that the different amounts of herb do not seem to affect gravity and pH.

Unfortunately the majority of the control beers were contaminated. Only three batches, Hops "Low", Hops "High" and Bog Myrtle "high" were free from contamination. Possible reasons for the spoilage could be inadequate cleaning and sterilization of demijohns or exposure to air during gravity checks. Therefore, it is difficult to come to any conclusions about the four batches that were already contaminated before the inoculation. However, it can be seen in figure 3 that all test organisms did grow in the fermented wort. Particularly the *L. buchneri* strain grew very well in the fermented wort and the beer with Marsh Tea, but in the beers with Bog Myrtle (Figs. 6 and 7) and Hops (Figs. 8 and 9) this strain seems to be inhibited.

The beers had not been filtrated and were therefore a bit cloudy. It would be expected that particles would settle in the bottom of the Duran bottles with time and therefore the optical density (OD) was expected to decrease with time for the control beer. Nevertheless, this was not the case for all the control beers and the results are therefore somewhat confusing.

Figure 6 demonstrates clear growth especially of *L. brevis*, this was also confirmed by the membrane filtrations for this beer, which were positive for all test organisms. The Bog Myrtle beer with high concentration seems to be a more hostile medium for the bacteria. Compared to Bog Myrtle beer with low concentration, the high concentration of Bog Myrtle inhibits *L. brevis.* Also, the plates from the membrane filtrations show only growth for *A. pasteurianus*. The results show that leaves of Bog Myrtle have antibacterial properties and that beers with higher amounts of this herb have stronger microbial resistance.

The hopped beer shows also microbial resistance against the three gram positive bacteria (Figs. 8 and 9). This was expected and is consistent with literature (Simpson 1993). However, one of the plates (High) had growth of four *P. damnosus* colonies. Furthermore, in the figures 8 & 9 it seems to be quite an increase in optical density for *P. damnosus*. The four colonies of

P. damnosus in one of the replicates of hopped beer (High) might indicate that Bog Myrtle confers higher microbial resistance to the brew, but this needs to be tested in a more elaborate study. The gram negative bacteria *A. pasteurianus* grew well in both of the hopped beers; the whole membrane was covered with colonies and the distinct smell of acetic acid was noticed.

In conclusion, *Ledum palustre* seems to have none or very little antibacterial activity when used in wort boil for 60 minutes, at least for these four common beer spoilage organisms. The fact that it was contaminated from the beginning implies that it had weak resistance to spoilage compared to the other beers. The results from the Bog myrtle beer show that it has resistance to bacteria, however, the "low" concentration of 3g/L was insufficient. Compared to the hopped beer, Bog Myrtle at "high" concentration seems to be a slightly better protection for the three gram-positive bacteria, especially for *P. damnosus*. The antimicrobial resistance of Bog Myrtle leaves requires further research. Especially to examine the long term protection against beer spoilage organisms and also investigate what components are responsible for the antibacterial activity. With more knowledge of the components, optimal addition times and concentrations could be established. Another interesting point would be to examine the combined effects of Hops and Bog Myrtle, perhaps this could provide even broader protection. This could be of interest for the micro-brewery industry or anyone with an interest in traditional beer production.

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APPENDIX

Fermentation

Gravity

DAY	V	HL	HH	SL	SH	PL	PH
0	40	40	40	40	40	40	40
1	22	24	24	22	24	19	20
2	10	12	11	14	14	12	14
3	10	11	10	12	12	11	10
4	10	10	10	10	10	10	10
5	10	9	10	10	10	10	10
6	10	9	9	10	10	10	10

pH

DAY	V	HL	HH	SL	SH	PL	PH
0	5.32	5.39	5.32	5.18	5.23	5.18	5.16
1	3.81	4.11	4.25	3.82	3.84	3.83	3.81
2	3.69	3.83	3.89	3.77	3.75	3.82	3.84
3	3.73	3.88	3.92	3.8	3.76	3.81	3.88
4	3.83	3.8	3.87	3.81	3.78	3.83	3.86
5	3.8	3.87	3.92	3.85	3.89	3.91	3.86
6	3.75	3.94	4	3.97	3.97	3.92	3.89

Optical density (OD) After innoculation

OD 600 nm

Kontroll	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
W	0.159	0.155	0.146	0.141	0.151	0.153
SL	0.157	0.158	0.147	0.151	0.157	0.163
SH	0.167	0.163	0.159	0.147	0.152	0.151
PL	0.165	0.164	0.161	0.138	0.144	0.141
PH	0.189	0.197	0.191	0.171	0.170	0.171
HL	0.261	0.283	0.232	0.165	0.240	0.168
HH	0.254	0.249	0.266	0.233	0.170	0.210

L. brevis	Day 2	Day 4	Day 6	Day 8	Day 10
W	0.154	0.154	0.149	0.152	0.156
SL	0.168	0.162	0.162	0.165	0.167
SH	0.170	0.177	0.164	0.167	0.168
PL	0.163	0.165	0.178	0.150	0.147
PH	0.196	0.200	0.197	0.199	0.190
HL	0.190	0.194	0.198	0.177	0.173
HH	0.221	0.236	0.230	0.226	0.219

L. buchneri	Day 2	Day 4	Day 6	Day 8	Day 10
W	0.150	0.144	0.171	0.157	0.159
SL	0.160	0.203	0.166	0.162	0.163
SH	0.158	0.157	0.175	0.168	0.168
PL	0.158	0.155	0.159	0.156	0.153
PH	0.185	0.187	0.193	0.189	0.189
HL	0.176	0.179	0.186	0.185	0.184
HH	0.206	0.229	0.240	0.239	0.248

P. damnosus	Day 2	Day 4	Day 6	Day 8	Day 10
W	0.145	0.141	0.161	0.153	0.159
SL	0.160	0.152	0.177	0.167	0.167
SH	0.154	0.153	0.156	0.163	0.179
PL	0.154	0.147	0.147	0.151	0.151
PH	0.181	0.192	0.188	0.196	0.189
HL	0.174	0.193	0.216	0.185	0.182
HH	0.203	0.275	0.242	0.239	0.234

A. Pasteurianus	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**
W	0.150	0.142	0.160	0.147	0.145
SL	0.160	0.155	0.165	0.162	0.155
SH	0.160	0.156	0.164	0.163	0.161
PL	0.178	0.154	0.162	0.155	0.152
PH	0.190	0.192	0.199	0.195	0.193
HL	0.178	0.216	0.197	0.186	0.183
HH	0.206	0.217	0.237	0.225	0.221