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Biochemical, microbiological and phytochemical studies on aqueous-fermented extracts from *Atropa belladonna* L. Part 1 – Biochemistry and microbiology

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Extraction methods of fresh plants into aqueous-fermented extracts according to German Homoeopathic Pharmacopoeia (HAB), regulation nos. 33 and 34 were evaluated. In the course of production, the extraction is accompanied by fermentation and the resulting preparation is stored for at least 6 months until further processing. The present work aimed at revealing the underlying biochemical reactions during manufacture and storage. In addition, the responsible microorganisms were isolated and identified. To study the robustness of the preparation method, formulation components as well as production conditions were varied. Additionally, questions were addressed at the reproducibility of the method and a comparison with an ethanolic extract was also performed. From 2006 to 2009, 110 extracts from the fresh flowering herb of *Atropa belladonna* var. *belladonna* (L.) were produced and analyzed. The results show that lactic acid bacteria (LAB) are primarily involved in the fermentation process, mainly producing lactic acid besides acetic acid and ethanol. The homofermentative *Lactobacillus plantarum* and the heterofermentative *Lactobacillus brevis* were identified as predominant lactic acid bacteria. Finally, factors for a successful fermentation are proposed.

1. Introduction

During the manufacture of herbal medicines the extraction of the starting material is the first step determining the compound spectrum of the resulting extract. Influencing production parameters are the use of fresh or dried plant material, the application of water or a lipophilic extraction medium as well as specific temperatures and extraction periods.

In contrast to phyto-extracts according to the Pharmacopoea Europaea (Ph. Eur.), homoeopathic manufacturing methods are usually based on fresh plant extraction, where ethanol or water may be used as extraction media (Biber et al. 2009; Ph. Eur. 6th edition: Mother tinctures for homoeopathic preparations). The manufacture of the so-called mother tinctures is laid down in the European Pharmacopoeia as well as in the national pharmacopoeias, i.e. in the German Homoeopathic Pharmacopoeia (HAB, Homöopathisches Arzneibuch).

Herbal mother tinctures can be adjusted to the content of constituents similar to the extracts for phytotherapy. Nonetheless, the quality is primarily characterized by the starting material used and a reproducible, defined manufacturing process (Ph. Eur. 6th edition: Mother tinctures for homoeopathic preparations). For this reason the methods of preparation include detailed information on the specific ratio of plant material to extraction medium as well as on maceration period and temperature conditions (Ph. Eur. 6th edition: Mother tinctures for homoeopathic preparations).

The aim of this work was to study the aqueous-fermentative extraction methods of the German Homoeopathic Pharmacopoeia (HAB 2003), regulation nos. 33 and 34 which were developed by empirical experience and established in the German Homoeopathic Pharmacopoeia in 1985 (HAB 1, 3rd supplement 1985) without any significant modification since then. While fermentation according to regulation no. 33 is performed using fresh plant by addition of honey, lactose monohydrate and purified water, no. 34 prescribes the addition of whey, purified water and in one case lactose monohydrate (HAB 2003, regulation no. 33, 34). The peculiarity is given by the changes of warm (37 °C or room temperature) and cold (2–8 °C, ice-water) temperatures as well as by the addition of ash. The latter is obtained from the dried plant residue and is added to the extract on the 7th day of manufacture. The mother tincture resulting matures for at least 6 months. Before further processing any sediment that may have been formed is removed and germ depletion is done by filtration (0.2 μm) or pasteurization. Each of the processes - extraction, fermentation and storage- shall influence the quality of the extract. So far, existing information is limited to phytochemical data on selected aqueous-fermented extracts. In this context, Millet studied herbal drugs of different compound classes, which were processed according to the methods of the German Homoeopathic Pharmacopoeia (HAB 2003), regulation nos. 33 and 34 and were subsequently stored up to 12 months. Aqueous-fermented extracts from *Allium cepa* (bulb: hydrophilic substances,

Table 1: Standard formulation according to HAB, regulation no. 33a

Name	Formulation (parts by mass)				(mg/100 g)
	Plant	Honey	Water	Lactose	Ash
Honey 0.75 (Standard)	100	0.75	50	0.75	50

flavonoids), *Arnica montana* (whole, flowering plant: sesquiterpene lactones), *Betula pendula* (leaves: flavonoids), *Gentiana lutea* (root: iridoids, xanthones), *Hypericum perforatum* (flowering herb: naphthodianthrones, phloroglucinols, flavonoids) and *Nicotiana tabacum* (leaves: alkaloids) were studied and a transformation of the genuine constituents could be observed (Millet 2010). *Inter alia*, a decline of nicotine in fermented tobacco leaves extracts (Millet et al. 2009) as well as a transformation of flavonoids in such from birch leaves (Millet et al. 2010) was reported.

However, the impact of the manufacturing technology on the extract quality as performed for herbal homoeopathic oil preparations by Heldmeier et al. (2009) has not been investigated yet. It still lacks a scientific consideration of the underlying biochemical and microbiological processes during the fermentation as well as of the influence of the manufacturing conditions on the phytochemical characters of the extract. Present knowledge assumes that lactic acid bacteria are involved in the fermentation and the resultant lactic acid is responsible for the stability of the extracts. This assumption is based on empirical experience as well as on observations showing a pH decline during manufacture (HAB 2003, regulation no. 33) and the fact that Millet could detect lactic acid bacteria on MRS agar (Millet 2010).

Therefore the aim of this work was to study the preparation of herbal mother tinctures from fresh flowering herb of *Atropa belladonna* var. *belladonna* (L.). As this drug is normally treated according to HAB, regulation no. 33a, that preparation method was defined as standard procedure and based on this, different extraction conditions were applied contrasting current practice. For comparative reasons the fermentation with whey addition according GHP, regulation no. 34c as well as ethanolic extraction were produced. Furthermore, the reproducibility was tested by preparing extracts parallelly and such with batch split. Microbiological, bio- and phytochemical results obtained should help to elaborate which production parameters are essential for manufacture and how an optimal method according to present knowledge might be defined.

In this first part of the work the biochemical reactions from aqueous-fermented extracts from the fresh flowering herb of *Atropa belladonna* var. *belladonna* (L.) were studied and the responsible microorganisms were identified. The phytochemical attributes of the extracts will be presented in part 2 of this paper series (Schwarzenberger et al. 2012).

2. Investigations, results and discussion

2.1. Appearance and odour of the extracts during manufacture and storage

Appearance and odour of the extracts changed during manufacture. The green cloudy liquid cleared and became brown during the maturation period. Due to fermentation the odour shifted from herbal to sour fruity. Spoiled extracts remained cloudy and exhibited a nasty odour. In contrast, the ethanolic extracts were characterized by an herbal odour over the whole maturation period.

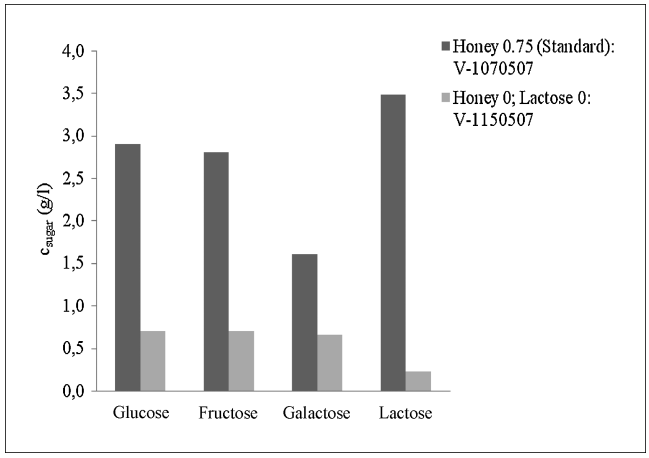

Fig. 1: Levels of monosaccharides and lactose in an extract with standard formulation (Honey 0.75) compared to a batch without substrate addition (Honey 0; lactose 0) at 3.5 days

2.2. Carbohydrate metabolism: Levels of glucose, fructose, galactose, sucrose and lactose in the aqueous-fermented extracts during manufacture and storage

As carbohydrates are substrates for lactic acid as well as ethanol fermentation, their levels were monitored during the fermentation process. The carbohydrates added according to HAB, regulation nos. 33 and 34 are honey, lactose monohydrate and whey. Honey is composed of water (17 %), fructose (38 %), glucose (31 %), maltose (7 %) and sucrose (2 %) (Belitz et al. 2007; Landis 1977). Whey usually contains 32–41 g/l of lactose and 0–0.4 g/l of galactose as predominant carbohydrates. Glucose and fructose as well as in low quantities galactose and lactose were detected in the *Atropa belladonna* plant juice. But as shown in Fig. 1 these carbohydrate levels are negligible in relation to the added amounts of the standard formulation (Table 1). The amounts of honey and lactose were varied in the so-called honey and lactose series to study their impact on fermentation. In addition to these batches other extracts also gave a view into carbohydrate metabolism.

As expected, all carbohydrates were metabolized during manufacture. The consumption of sucrose, glucose and fructose started in the first 7 days whereas sucrose was detectable at best

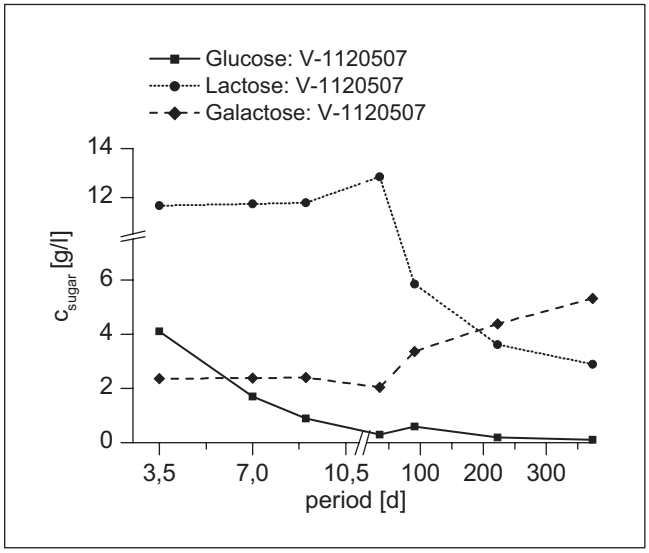

Fig. 2: The variant Lactose 2.5 exemplarily shows stable levels of lactose over 36 days and even a later increase of galactose as opposed to the fast glucose degradation

in the first 9 days as opposed to the other carbohydrates. In contrast, the levels of lactose and galactose usually remained stable in the same period and their decrease revealed at a later date as exemplarily shown in variant Lactose 2.5 (Fig. 2). The results can be explained by the direct utilization of the monosaccharides glucose and fructose following glycolysis whereas for lactose metabolism it must be first hydrolyzed by β-galactosidase to glucose und galactose (Kandler 1983). After hydrolysis galactose was metabolized at a much smaller rate than glucose. When lactose degradation had started, galactose concentration increased in some extracts (honey 2.5, lactose 2.5 and honey 0.75; 7 d; 2–8 °C) as shown exemplarily in Fig. 2 while glucose levels remained low. This finding can be explained by the necessity of the enzymatic conversion of galactose to glucose for utilization (Kandler 1983). In particular the necessary enzymes are only induced in times of nutrient lack but are suppressed by high glucose concentrations (Voet et al. 2002). Further confirmation was obtained when batches without honey addition were monitored. As in those cases lactose was the sole carbohydrate source, its degradation already started in some extracts in the first days. Furthermore, in extracts with high amounts of substrate (honey 2.5 und lactose 2.5), the decrease of lactose and galactose was not detected until the consumption of the easily digestible glucose and fructose. In these tests the low pH and bacterial counts at high lactose and galactose levels after 5 weeks also proved that the low pH and not a lack of substrates is responsible for stopping the fermentation. These results agree with observations in a study on vegetable juices fermentation (Gardner et al. 2001). In extracts prepared by whey addition the concentration of lactose and galactose remained stable or decreased just a little in the first 7 days. So it can be concluded that the starter cultures, which were used in the course of whey, were not able to grow on in the herbal environment. In particular, the cultures from dairy industry are specialized in lactose as substrate.

The batches with varying temperatures revealed that carbohydrates decreased the faster the higher the temperature was. This finding can be explained by the better growth conditions for microorganisms at higher temperatures.

The reproducibility of the procedure was proven with similar courses of sugar degradation in parallelly manufactured extracts and such with batch split after 7 days. The same results were observed in the tests with varying amounts of ash which were also split before addition of the ash. So it can be concluded that ash addition does not have any influence on sugar metabolism.

2.3. Acid metabolism: pH of the aqueous-fermented and ethanolic extracts during manufacture and storage

The pH is an important parameter of aqueous-fermented extracts for in-process control during manufacturing and quality control of the final extract. The desired lactic acid bacteria are acidophilic in contrast to spoilage microorganisms (Buckenhüskes et al. 1986; Jay et al. 2005). Therefore the specification of *Atropa belladonna* ex herba ferm extract is set from 3.0 to 4.5 as for the most aqueous-fermented extracts and a fast acidification during manufacture is desirable. The microbiological data of this work confirmed that a low pH value inhibits the growth of spoilage microorganism (see 2.6.). Fermentation usually starts within the first 7 days, wherefore the pH was determined twice a day in this period. During the storage, pH was assessed out after 1 day after filling, after 5 and 13 weeks as well as after 7 and 12 months. The pH decrease can be explained by the formation of lactate and acetate because their increase paralleled (see 2.4.).

It was shown that lower pH values were reached during the maturation period by increasing the substrate levels (Fig. 3). Batches with a little less sugar (honey 0.5 and lactose 0.5) were similar to

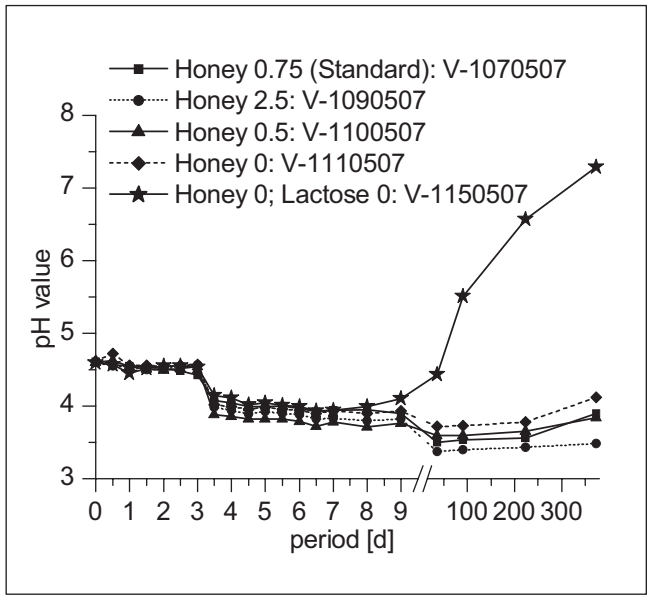

Fig. 3: Different pH courses depending on the respective honey amount added (honey series 1) during the maturation period

the standard whereas extracts with small sugar amount (honey 0 and lactose 0) had a higher risk to spoil during storage. The pH of the batches without carbohydrate addition exceeded the upper specification limit in all cases after 13 weeks at the latest (honey 0; lactose 0, Fig. 3). The results can be explained by a higher lactate formation by increasing substrate levels corroborating earlier results in food such as sauerkraut and pickled cucumbers (Holzapfel 1996; Krämer 2007). Therefore it can be summarized that by the addition of carbohydrates the production risk can be reduced. As described in 2.2., honey addition is preferable due to the direct availability of glucose in comparison with whey or lactose.

Because of the low pH of the added acid whey, such tinctures showed a lower pH (< 4.5) at the beginning of maceration compared to extracts with honey. In this way, optimal proliferation conditions for the acidophilic LAB were provided.

By adding *Lactobacillus plantarum* as a starter culture the acidification started one day earlier compared to the standard whereby the pH values from both variants were similar after 7 months. The faster acidification is plausible due to the higher number of LAB which do not need to compete with the wild flora and can directly start with formation of lactate. As the same substrate amount was added in both variants similar pH resulted. These results comply with researches about culture application in sauerkraut production and vegetable juices fermentation (Gardner et al. 2001; Halász et al. 1999). Furthermore, by addition of a starter culture the natural variability of the genuine flora, e.g. due to a previous rain period, can be compensated. Working with starter cultures is also known from preparation of silage because they increase the manufacturing safety (Pahlow and Honig 1986). In the same line, yeasts are added during the preparation of spagyric tinctures according to Strathmayer to induce an ethanolic fermentation (HAB 2003, regulation no. 50a/b).

If the extracts did not acidify strongly enough within the first 7 days, the addition of ash (50 mg/100 g) could increase the pH for a short time. Upon continuing fermentation, however, the pH decreased again. This result was especially noticeable in the cold-macerated batches when the fermentation started not until the end of the 7-day-storage-period in the cold (Fig. 4, see below). It was thus proven that with an adequate acidification the application of ash within the range tested did not exert any

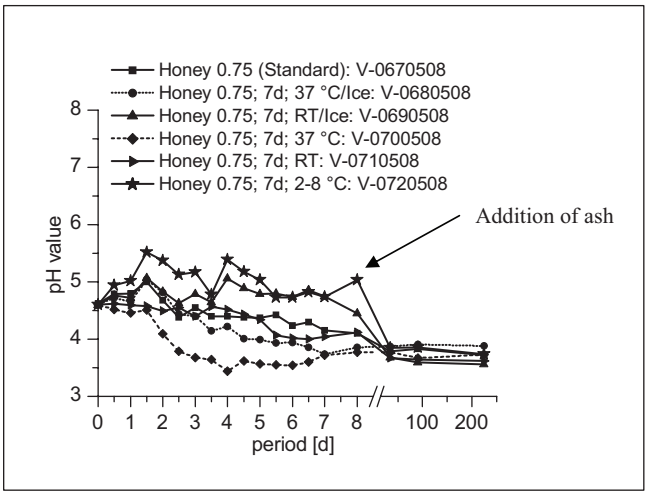

Fig. 4: Different starting points of acidification in the extracts at varying maceration temperatures (temperature series 1) and further development during the maturation period

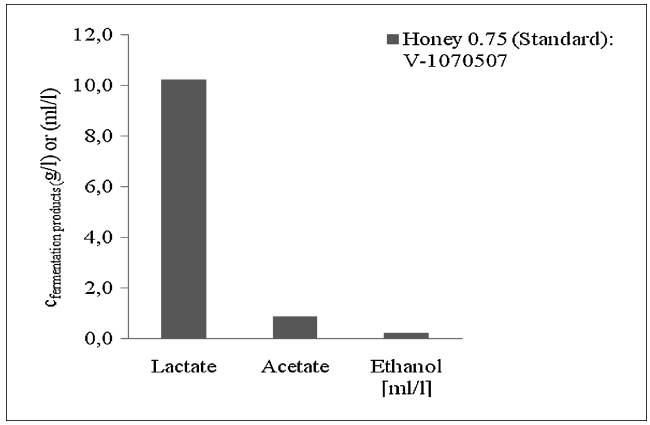

Fig. 5: Levels of the predominant fermentation products in an extract with standard formulation (honey 0.75) at 7 months

influence on the pH of the extracts during maturation. The pH values remained stable even increasing the amount by 50 % (honey ash 75 and whey ash 75). However, if adequate acid levels were not produced, the application of ash could disturb the pH balance of the extract. This was demonstrated with the ash series prepared in 2006 with plant material harvested late. In both systems (honey and whey) the pH values exceeded the upper pH limit. Only the tincture honey ash 0 remained stable, which was prepared without the addition of ash. Consequently, the ash amounts and the pH values correlated in this series. This result can be explained by a lack of acids in these extracts which would have been able to buffer the basic ash. Confirmation was also obtained by a further test where the application of 50 mg herbal ash to 100 g purified water resulted in a pH > 10. So it can be concluded that ash application only has a negative influence, if there is a lack of acids that should be avoided because of stability reasons.

By using plant material harvested at a later date, the pH exceeded the upper limit during the maturation period in 92 % of the cases. This may be explained by the fact that the LAB flora was not vital enough, e.g. due to a preceding dry summer.

By pressing the macerates earlier than the standard, acidification started later (honey 0.75; pressing after 3 h, 0.5 d and 1.0 d) but the reached values were similar after 7 months. The result can be explained by the earlier separation of the microflora adhering to the herbal material and thus, the current practice of HAB pressing after 3.5 days maceration is confirmed.

The tests with varying maceration temperatures showed that the acidification started earlier at a constant temperature of 37 °C (honey 0.75; 7 d; 37 °C; Fig. 4) compared to the standard. Generally, extracts with cooling phases acidified more slowly than such without (Fig. 4). After 7 months, the pH values of all trials were sufficiently low irrespective of the maceration temperature. The faster acidification by increasing the temperature can be explained by the better growth conditions of lactic acid bacteria with an optimum of 30 °C (Jay et al. 2005; Ternes 2008). These results are in line with studies on sauerkraut fermentation (Halász et al. 1999).

The negative controls of all test variants were the trials with access of oxygen which spoiled, associated with a strong pH rise. As lactic acid bacteria are anaerobes, in the manufacture of sauerkraut and silage one takes care of the absence of air prior to production. Therefore the strong pH increase can be explained by aerobic germs such as yeasts and moulds encountering better growth conditions than the desirable LAB (Holzapfel 1996; Kessler et al. 2010; Pahlow and Honig 1986).

In the course of the maturation period the pH of the successfully fermented extracts continued to decrease whereby the minimum was reached after 5, rarely after 13 weeks. From then on, the pH values remained stable or even slightly increased. These courses are known from the sauerkraut fermentation where fermentation is also finished after 5 weeks (Ternes 2008). The reproducibility of the method was proven with parallelly prepared trials and such with batch split after 7 days. The first showed a similar pH course, the second an identical one.

In 23 of the 106 prepared aqueous-fermented tinctures pH exceeded the upper specification limit of 4.5 during the maturation period. 15 of these extracts were already out of specification and partly began to spoil after 13 weeks. Eight extracts exceeded the limit at a later date without signs of spoilage ("creeping increase"). The causes were found for 19 batches where worst case conditions such as lack of substrate (honey 0; lactose 0, Fig. 3), late point of harvest and access of oxygen were responsible (Table 2). Only for 4 extracts in 2009, produced according to the standard method with optimal starting material, no reasons for spoilage could be found. Probably this is attributable to the variability of the microbial flora on the plant. As a consequence, the dependence of the quality from starting material described by Biber et al. (2005) for ethanolic mother tinctures is also true for aqueous-fermented extracts.

With the exception of the extracts with a creeping increase, fermentation was generally finished after 13 weeks: Either the extracts were successfully fermented or failed by exceeding the upper pH limit. Based on these data the 13-weeks-rule was postulated. As the tinctures with a creeping increase were also sufficiently acidified after 13 weeks, it can be concluded that an excessively long storage after fermentation may have a negative influence on the extract stability.

In contrast to the aqueous-fermented extracts the ethanolic ones showed a constant pH of 5.4–5.6 throughout the manufacturing and storage period. As expected no fermentation was found.

2.4. Acid metabolism: Concentration levels of D*-lactate,* L*-lactate and acetate in the aqueous-fermented extracts during manufacture and storage*

Depending on the fermentation type (homo- or heterofermentative lactic acid fermentation, ethanol fermentation) carbohydrates may be metabolized into lactate, ethanol, acetate and CO_2. D- and L-Lactate are the main products of the homofermentative lactic acid fermentation (Kandler 1983). In addition to lactate, acetate may be formed during heterofermentative lactic acid fermentation (Kandler 1983) and in the course of spoilage (Lyberatos and Skiadas 1999). The acid levels in the

Table 2: Aqueous-fermented extracts showing pH values above 4.5

Extract	Number	Point of pH increase	Cause
Honey 0.75 (Standard)	1	after 5 or 13 weeks	2006: Late point of harvest
	4		2009: Cause not surely known
Whey	1	after 5 weeks	Late point of harvest
Honey 0.75; Oxygen access	2	after 13 weeks	Access of oxygen
Honey 0; Lactose 0	4	after 1 day to 13 weeks	No addition of carbohydrates
Lactose 0	2	after 7 to 12 months	No addition of lactose
Ash extracts (honey and whey)	9	after 5 weeks to 7 months	Late point of harvest
Sum	23		

aqueous-fermented extracts were determined to obtain a deeper understanding of the fermentation process.

D- and L-Lactate were neither detected in the plant juice nor in the extracts sampled immediately after preparing the macerate. Lactate was primarily found after 1.5 days allowing the conclusion that its formation during manufacture is exclusively attributable to lactic acid fermentation. Either similar amounts of both enantiomers or more D-lactate were formed. The formation of D- and L-lactate can be ascribed to the presence of the isolated lactic acid bacteria (see 2.6.) which are able to build both enantiomers (Liu 2003). The results confirm the assumption that the manufacture of aqueous-fermented extracts is governed by lactic acid fermentation and that the lactic acid formed is responsible for the pH decrease and the extract stability.

Acetate was detectable in the plant juice and was also generated during manufacture. In most cases its formation can be attributed to heterofermentative lactic acid fermentation, because the acetate increase paralleled lactate formation. However, nonparallel courses, e.g. in the trials with oxygen access or in the tests without substrate addition, point to spoilage process (Eichholtz 1975; Lyberatos and Skiadas 1999). Due to the fact that lactate was the main fermentation product (Fig. 5) it can be concluded that homofermentative lactic acid bacteria dominated. In case of heterofermentative fermentation equal amounts of lactate and acetate would have been detected.

Extracts showing pH values out of specification contained low concentration levels of lactate. For sauerkraut a lactate concentration of 6.0 g/l is necessary for microbiological stability (Belitz et al. 2007). In this study most of the unstable extracts showed a lower content. On the other hand there were stable batches also exhibiting lower lactate concentrations. Consequently, extracts with similar pH values did not necessarily contain equal acid contents. This observation can be explained by the varying buffer capacity of the plant which depends on the amount of the base atropine in the case of *Atropa belladonna*. This fact is also known from sourdough production and is attributed to the varying buffer properties of the flour (Spicher and Stephan 1999).

Higher lactate concentrations were regularly formed by increasing the amount of substrate as shown in Fig. 6. This positive correlation is also known from sauerkraut production where acid amounts depend on the sugar content of cabbage (Belitz et al. 2007). Interestingly, the amounts of D- and L-lactate did not always correlate with the substrate concentrations. An explanation could be the natural variability and diversity of the microflora. The variants without substrate addition (honey 0; lactose 0) exhibiting a pH out-of-specification, formed less lactate and showed lactate degradation during the storage period (Fig. 6). Furthermore, these extracts contained higher acetate concentrations than the standard in 3 of 4 batches which may be attributable to a spoilage process. Therefore it can be concluded that the genuine plant sugars are not sufficient to prepare a stable extract. With regards to the variable buffer capacity (see above), a possible proposal for optimization could be the flexible addition of substrate dependent on the individual fermentation process, which, however, was not subject of this work. At this moment the substrate amounts are fixed by the formulation of the HAB. So-called "refeeds" known from biotechnology are not considered, yet (Dellweg 1994; HAB 2003, regulation no. 33).

The added whey usually contains 1.8–2.0 g/l D-lactate and 6.0–8.7 L-lactate. The high content of the L-enantiomer is attributable to the used starter culture from dairy industry for whey preparation where cultures producing more L-lactate are preferred because of an improved human physiologic utilization (Liu 2003). Since after 3.5 days the D-/L-lactate-ratio in the whey extracts was about 1, it can be assumed that the whey cultures could not proliferate in the herbal environment. In this case the L-lactate level would have been higher.

In the extracts with added starter cultures the formation of lactate and acetate continued until 7 months as opposed to the other batches. These extracts contained more acetate after 7 months than the standard although they were prepared with the homofermentative *Lactobacillus plantarum*. This observation points to the presence of a heterofermentative lactic acid bacterium beside of the used starter culture.

The successfully fermented ash series showed parallel courses in increasing and decreasing trends of lactate and acetate, independently from the added ash amount as exemplarily shown in the case of L-lactate in Fig. 7. In contrast, lactate was degraded in the spoiled ash series manufactured with starting material harvested late.

The use of washed plant resulted in lower lactate concentration explainable by washing off the natural lactic acid bacteria flora.

The tests with varying maceration temperatures showed that acidification started earlier when fermentation was performed at higher temperature levels (honey 0.75; 7 d; 37 °C > honey 0.75; 7 d; 37 °C↔ice > honey 0.75 (Standard)). The trails honey 0.75 (Standard) and honey 0.75; 7 d; 37 °C↔ice formed the highest acetate levels in this series whereas a constant maceration at 37 °C induced low acetate concentration. The earlier start of lactate formation can be explained by the optimal growth conditions for LAB complying with the results of carbohydrate degradation and pH courses. The maceration at 37 °C yielded low acetate values because homofermentative lactic acid bacteria were selected at this temperature (Holzapfel 1996). This fact is also known from studies of sourdough production where at warm conditions (30–35 °C) lactic acid is preferentially formed by *Lactobacillus plantarum* and under cold conditions *Lactobacillus brevis* (20–25 °C) induced higher levels of acetate (Belitz et al. 2007; Spicher and Rabe 1981; Spicher and Stephan 1999). Most remarkably, these two lactobacilli were often found in the extracts (see 2.6.).

The similar or identical courses of lactate (Fig. 7) and acetate in the parallel and batch split extracts demonstrate that the quality of fermentation primarily depends on the starting material with a varying natural microflora as well as on the production conditions within the first week.

During maturation, lactate and acetate formation proceeded. The maximum of lactate was reached after 5 to 13 weeks with the

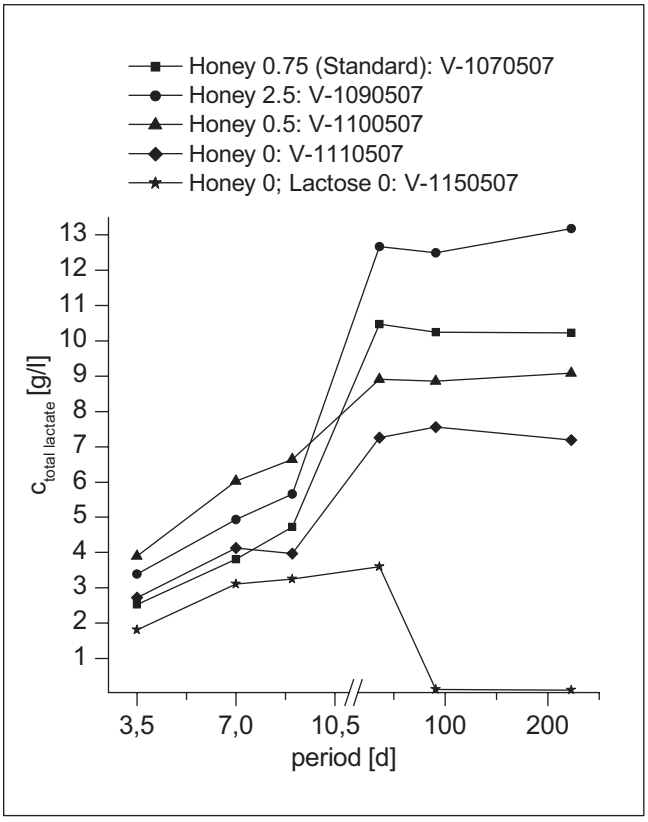

Fig. 6: Different levels of lactate depending on the honey amount added (honey series 1) during the maturation period

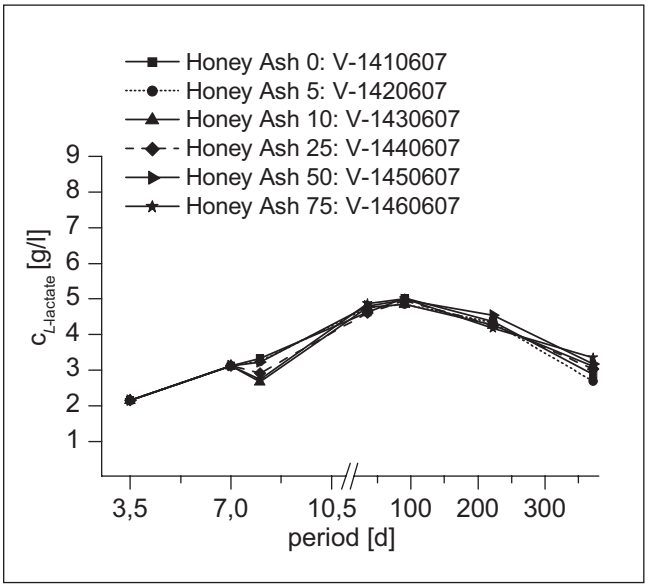

Fig. 7: Similar courses of *L*-lactate levels in extracts despite varying amounts of ash (ash series honey 1) during the maturation period

exception of extracts prepared with starter cultures (see above). Then the contents remained stable or decreased whereas in the stable extracts the lactate decline did not notably start until 7 months of storage (Fig. 7). In contrast, the concentration of acetate decreased much faster than lactate during storage. Tinctures quickly turning into spoilage showed a lactate decrease already in the first 13 weeks and upon oxygen access lactate was degraded rapidly. These results confirm the 13-week-rule postulated in context with the pH development.

The degradation can be explained by successive cultures whose growth is promoted by oxygen access. Especially yeasts like *Candida* isolated in this work (see 2.6.) are able to metabolize lactate resulting in a pH decrease (Dellweg 1994; Holzapfel 1996). In this context it must be mentioned that during the study the air space in the bottles increased because of the continual sampling. In this way the process shall be promoted in comparison without sampling (see 2.6.). In summary, the results on lactate and pH course show that an excessive long storage after fermentation may have a negative impact on the extract stability. This conclusion agrees with knowledge from pickles production where the products are airtightly packed and pasteurized to prevent successive cultures and to reach a long shelf life (Holzapfel 1996). Therefore the 6-months-storage of the HAB (HAB 2003, regulation no. 33–37, 51), prescribed for all plants, should be reconsidered. The 13-weeks-rule postulated should be verified in this context, because no sampling was performed between 5 and 13 weeks during this work and the kinetic of the lactate formation points to an earlier end of the fermentation process. Other preparation methods of the HAB do not define a period, but storage until the end of fermentation is mandatory (HAB 2003–2008, regulation no. 21, 22, 25–30, 47). This formulation respects the natural variability of the fermentation process.

2.5. Concentration levels of ethanol, methanol, 1-propanol, 2-propanol, acetaldehyde, acetone and ethyl acetate in the aqueous-fermented extracts during manufacture and storage

Ethanol can be formed from carbohydrates by heterofermentative lactic acid fermentation (Kandler 1983) as well as by ethanol fermentation (Heider 2006; Jay et al. 2005). Methanol may be a result following hydrolysis of the plant's pectin esters (Wucherpfennig and Bitsch 2004). Furthermore, the formation of acetaldehyde, acetone, ethyl acetate, 1-propanol and 2-propanol in aqueous-fermented extracts is possible by failed fermentation (Bruchmann 1976). Against this background, the concentration levels in the aqueous-fermented extracts were determined to obtain an insight into the fermentation process.

Analysis of the fresh plant juice revealed up to 1.1 ml/l methanol and lower levels of ethanol while all other substances were not detected.

The concentration of methanol in the extracts was independent of the manufacturing conditions and the fermentation process with the following exceptions: spoiled extracts following oxygen access showed a degradation of methanol after this incident as well as washing the plant and earlier pressing reduced the methanol content. In the other extracts similar concentration levels were determined within the series as exemplarily shown in Fig. 8. Consequently, methanol formation during manufacture of aqueous-fermented extracts can be exclusively attributed to the saponification of the pectin esters during the extraction period (Comment to Ph. Eur. suppl. 6.1: Extracts). This was proven by methanol detection in the plant juice, the independence of methanol concentration from the production conditions and the fermentation process as well as by reaching similar concentrations in the same series. The lower levels when pressed earlier can be explained by the shorter contact time of the pectin methylesterase with the plant material. Through washing of the plant material, these enzymes could be rinsed off and thus reducing the methanol formation. Probably methylotrophic bacteria and yeasts (Chistoserdova et al. 2009; Sahm and Wagner 1972) were responsible for methanol degradation in the batches with oxygen access. As methanol concentration did not increase during maturation, methanol formation by microorganisms and failed fermentation can be excluded during the evaluated manufacturing method. The specification limit of the HAB for *Atropa belladonna* ex herba ferm 33a of 0.10 % (V/V) was always met (HAB 2009, Supplementary rules concerning the general mono-

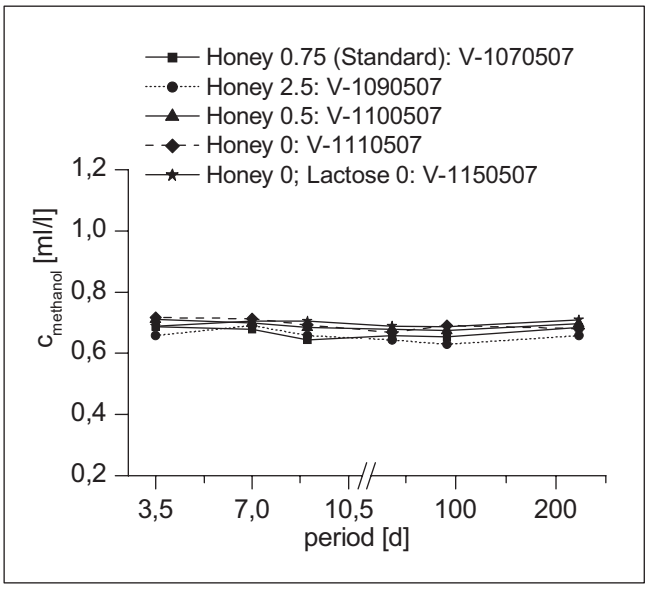

Fig. 8: Similar courses of methanol levels in extracts despite varying amounts of honey (honey series 1) during the maturation period typical minimum

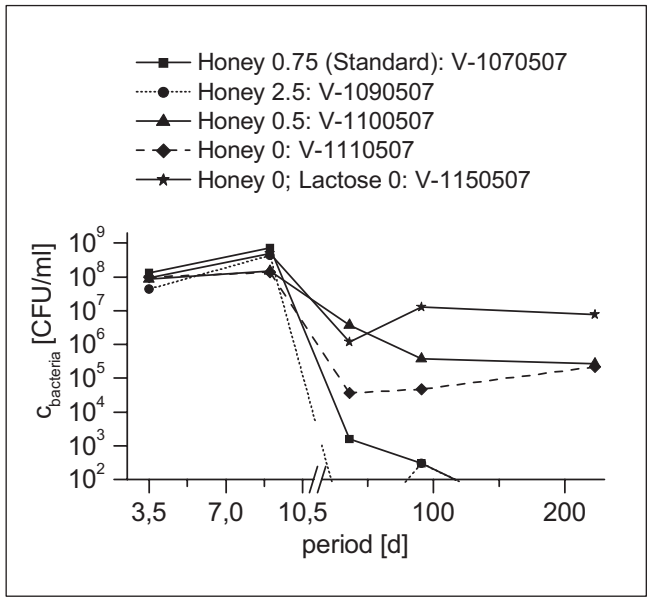

Fig. 10: Different bacterial growth curves in extracts with varying amounts of honey (honey series 1) during the maturation period

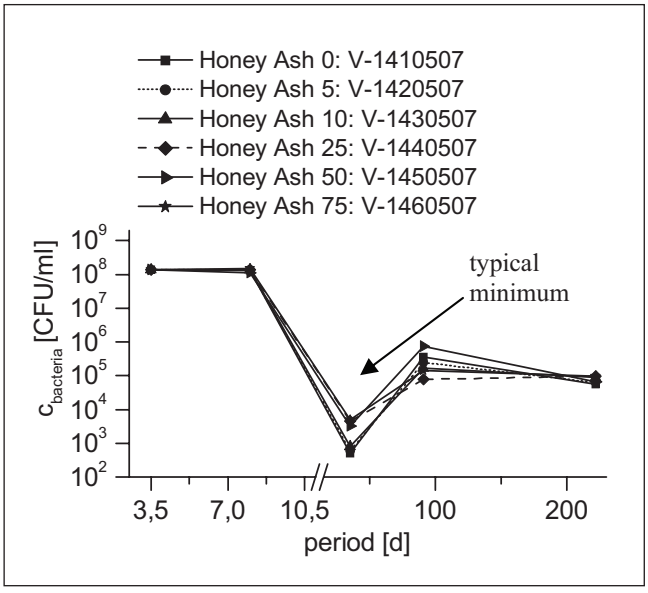

Fig. 9: Similar bacterial growth curves in extracts despite varying amounts of ash (ash series honey 1) during the maturation period with the characteristic minimum after 5 weeks

graph "Mother tinctures for homoeopathic preparations" of the European Pharmacopoeia).

In 87 of the 100 tested extracts ethanol concentration increased up to the detection limit during manufacture and storage. Formation started in the first days and continued up to 12 months. Notably, extracts with added starter culture did not form any ethanol.

A formation of ethanol in the first days of manufacture with constant levels in the further process can be attributed to the heterofermentative *Leuconostoc mesenteroides* known from sauerkraut production. While its presence was confirmed on the plant, it was not detected in any of the extracts which may be attributable to the fact that it stops growing at a pH below 4.0 (Gardner et al. 2001). Consequently, yeasts must primarily be responsible for ethanol formation in the further manufacturing process because the lactic acid bacteria isolated from the *Atropa belladonna* extracts (see 2.6.) do not possess this metabolic activity. This assumption was also confirmed by the observation that the added starter culture *Lactobacillus plantarum* was able to suppress yeast growth (see 2.6.) and ethanol formation. Low ethanol concentration resulted when incubation was performed at 37 °C because the homofermentative lactic acid bacteria more strongly profit from higher temperatures compared to heterofermentative strains and yeasts (Belitz et al. 2007; Holzapfel 1996). As shown in Fig. 5 far less ethanol compared to lactate was formed implying a stronger lactic acid rather than ethanol fermentation. In consistence with the lactate-acetate-ratios these results also proved that at the point of greatest nutrient supply, homofermentative were predominant compared to heterofermentative lactic acid bacteria.

Acetaldehyde was not detected in any of the tinctures whereas acetone, 1-propanol, 2-propanol and ethyl acetate were occasionally determined at up to 200 ppm. The data show that the formation of ethyl acetate can be attributed to the simultaneous presence of ethanol and acetate. Anyway the sporadic occurrence of these substances and their low quantities allow the conclusion that failed fermentations can be excluded when applying the manufacturing method according to HAB.

2.6. Microbiological aspects: Microbial count and specified microorganisms in the aqueous-fermented extracts during manufacture and storage

The preferred formation of lactic acid during manufacture (see 2.4.) suggested that lactic acid bacteria are primarily involved in carbohydrate degradation. On the other hand the formed ethanol (see 2.5.) pointed to the presence of yeasts. To get an overview of the fermentation process, the microflora of the aqueous-fermented extracts was investigated.

Bacteria, yeasts and moulds were detected in the fresh plant material. All fermented extracts showed a bacterial growth whereby the maximum was reached after 3.5 to 9 days. From then on the counts decreased and a minimum was often reached after 5, rarely after 13 weeks. Subsequently, the counts either remained stable or in most cases increased again (Fig. 9). Extracts with high amounts of honey (honey 0.75 (Standard) and honey 2.5) showed a typical exponential growth and death phase as exemplarily shown in Fig. 10. In contrast to this, bacterial counts remained at a high level when no substrate was

present (honey 0; lactose 0, Fig. 10). In tinctures with oxygen access the counts reincreased rapidly after this incident.
The high levels of bacteria in extracts with pH values out-of-specification (Honey 0; lactose 0 and honey 0.75; oxygen access) proved the preserving character of a low pH. As a consequence, in successfully fermented extracts (e.g. honey 2.5, Fig. 10) bacteria died off due to a low pH and not due to a lack of substrate which is in accordance with literature (see 2.2., Gardner et al. 2001). The reincrease of bacteria after a few months can be explained by successive cultures. In this context the rapid increase by access of oxygen points to aerobes. As described earlier (Jay et al. 2005) the low pH also inhibited the growth of spoilage microorganisms in this study. This was proven by the fact that *Pseudomonas aeruginosa* and *Staphylococcus aureus* were not found in any of the extracts. Enterobacteria were detected in the plant material and in some cases in the first 8 days of production, e.g. in the cold macerated extract that did not ferment in this time period (honey 0.75; 7 d; RT↔ice). After 5 weeks, however, they were no longer detectable, attributable to the preserving character of the low pH.
Yeasts were detected in 97 % of the aqueous-fermented extracts. In contrast to the growth curves of bacteria the yeast counts remained stable or slightly decreased during the maturation period or even increased. To obtain more detailed information the most frequently detected yeast colonies out of 48 extracts were examined: In this way *Candida* species (*C. krusei*, *C. kefyr*, *C. lusitaniae*, *C. inconspicua/norvegensis*) and in one case *Aureobasidium* ssp. (honey 0.75; oxygen access) were identified. In 79 % *Candida krusei* was determined and, consequently, it can be concluded that the yeast flora was independent from variations of the formulation and the respective production conditions. It is worth mentioning that the addition of a starter culture (*Lactobacillus plantarum*) completely suppressed yeast proliferation. Their growth was promoted by incomplete filled up bottles due to limited batch size as well as by continual sampling in this study. This problem is also known from wine production (Hühn et al. 1999). In this context it becomes clear that yeast counts did not decrease during the maturation period as they were successive cultures of lactic acid bacteria. At this juncture it must be pointed out that an excessive yeast growth is undesirable because they compete with the lactic acid bacteria for the substrate. Moreover yeasts are able to metabolize lactic acid thereby increasing the pH (Gardner et al. 2001; Ternes 2008), being especially true for the dominating *Candida* species (Dellweg 1994; Holzapfel 1996). With this knowledge it becomes clear, why lactate was also slightly degraded in stable extracts (see 2.4.).
In contrast to enterobacteria, yeasts as well as moulds can grow at low pH values (Jay et al. 2005). Consequently, in sauerkraut manufacture special care is taken to exclude air (Holzapfel 1996). In the present study the growth stimulating effect of oxygen on yeasts as well as on moulds (see below) was demonstrated by the spoiled batches with oxygen access in which these microbial counts increased rapidly.
In routine production storage containers should therefore be filled up. As the sampling of great volumes in this study should be regarded as worst case, yeast growth and concomitant lactate degradation can be excluded under normal production conditions. However, in the first 7 days the maceration mash and the extracted liquid are stirred. Doing so, oxygen is brought in and a competition between lactic acid bacteria and yeasts cannot be ruled out in this period. Therefore variants with air-less treatment during the first week should be considered in further studies. As the addition of starter culture was able to suppress yeast growth completely despite airspace in these bottles, it can be summarized that the addition of starter culture and the strict exclusion of oxygen are the most efficient measures to suppress undesired yeast contamination in the manufacture of aqueous-fermented extracts.
Moulds were detected in three extracts during the first 8 days of manufacture. After 5 weeks they were no longer present, attributed to reduced oxygen supply after filling. During maturation 96 % of all the aqueous-fermented extracts (n = 106) did not show any mould growth. Only in two stable extracts the mould count increased in this period. These individual cases can be judged as outliers because in the same variants of other series no moulds were found. Distinct mould growth with a microbial count up to 10^5 CFU/ml only occurred in the negative control of the spoiled extracts due to oxygen access. As a result, it can be summarized that mould contamination can be excluded in the manufacture of aqueous-fermented extracts.

2.7. Microbiological aspects: Concentration of bacterial endotoxins in aqueous-fermented extracts during manufacture and storage

Gram negative bacteria such as enterobacteria pertain to the natural microflora of plants. Their cell wall encloses lipopolysaccharides which are released as endotoxins after cell death (Jay et al. 2005). Endotoxins cannot be removed by common filtration (0.2 μm) or pasteurization before further processing the extract to a drug. Therefore endotoxin formation should be prevented in the manufacture of extracts.
Endotoxin levels in the tinctures were comparable after 8 or 9 days and depended on the natural contamination of the starting material. During the maturation period of 7 months, they were reduced with exceptions by at least 65 %, whereas extracts of the same series showed similar values. An increase of endotoxins was only detected in the extracts with oxygen access and in one successfully fermented batch from the ash series. The latter can be judged as outlier because no exceptional microbiological results were found compared with other extracts. In contrast to this, the high endotoxin concentrations in the cases of oxygen access can be explained by a massive microbial count.
The results demonstrate that under usual production conditions endotoxins cannot be formed during fermentation but are rather brought in by the natural microflora of the plant. As their level usually decreased during the maturation period, a growth of gram negative bacteria can be excluded. This goes along with the quick acidification observed because the growth of gram negative bacteria is inhibited at low pH values (see 2.6., Weber 2003). Therefore the exclusion of air and a quick acidification are preconditions to exclude endotoxine release during the manufacture of aqueous-fermented extracts beyond the natural contamination of the starting material.

2.8. Microbiological aspects: Lactic acid bacteria in the aqueous-fermented extracts during manufacture and storage

The identification of lactic acid bacteria was performed to allow conclusions on the exact biochemical processes governing the production of the aqueous-fermented extracts.
From the fresh plant material *Leuconostoc mesenteroides*, *Lactobacillus curvatus* and *Weissella minor* were isolated. At least 10^6 CFU/ml lactic acid bacteria were determined in the aqueous-fermented extracts after 8 or 9 days. It was demonstrated that LAB represented a great part of the total bacterial count in the first days of manufacture. This result agrees with the conclusion drawn due to high lactate concentration that primarily lactic acid bacteria are involved in fermentation of the evaluated extracts

(see 2.4.). As mentioned the low pH following acidification inhibits the growth of other concurring bacteria.

The lactic acid bacteria of the extracts were identified in most cases as *Lactobacillus plantarum* and *Lactobacillus brevis*. *Leuconostoc mesenteroides* was not detected in any extract although it is frequently met on plants (Krämer 2007) as it stops proliferating at pH $\leq$ 4.0 (Gardner et al. 2001). After 8 or 9 days *Lactobacillus plantarum* dominated whereas after 7 months in 85 tested batches different types of *Lactobacillus plantarum* (2 types) and *Lactobacillus brevis* (3 types) were detected in approximately equal quantities. Moreover, *Lactobacillus casei*, *Lactobacillus rossiae* and *Enterococcus faecalis* (honey 0.75; oxygen access) were occasionally determined. Furthermore, there were four isolates identified as a kind of *Lactobacillus* which could not be more closely assigned to a known species but show relationships to *Lactobacillus plantarum* and *Lactobacillus crustorum* respectively (personal communication, Prof. Dr. Ehrmann, Technical Microbiology, Technical University Munich, Germany).

The results obtained are in line with findings on sauerkraut production where in the first fermentation stage homofermentative lactic acid bacteria such as *Lactobacillus plantarum* dominate, being replaced by heterofermentative acidophilic *Lactobacillus brevis* in the further process (Halász et al. 1999; Krämer 2007). The mixtures of D- and L-lactate determined in the extracts (see 2.3.) correspond with the ability of both species to form both enantiomers. The dominance of the homofermentative *Lactobacillus plantarum* at the point of highest nutrient supply agrees with the conclusions drawn on the lactate-acetate-ethanol-ratios (see 2.4., 2.5.), pointing to a dominance of homofermentative lactic acid fermentation.

The slow degradation of lactose in extracts prepared with whey as well as the low concentration of L-lactate implied that the commercial starter cultures from the dairy industry (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus helveticus*) could not proliferate in the herbal environment. This assumption was confirmed by microbiological testing when none of these species was detected after 8 days or after 7 months in extracts prepared with whey. An explanation could be that starter cultures are adapted for milk as substrate. This result refutes a previous assumption whereupon starter cultures of whey are responsible for fermentation (Millet 2010).

Lactobacillus brevis was also isolated from extracts prepared with *Lactobacillus plantarum* as starter culture after 7 months. This result confirms the assumption due to high acetate concentration in these batches that heterofermentative LAB must be present (see 2.4). Moreover the findings show that the natural microflora is even involved in fermentation. This again agrees with knowledge from sauerkraut fermentation where a secondary fermentation by the wild flora despite the application of starter culture could be observed during storage (Buckenhüskes et al. 1986). Due to the unsterile production conditions of the HAB method the participation of a wild flora must always be considered whereas the risk for its proliferation increases with extended storage (see below).

The homogeneity of the results from all batches proves the independency of the microbial flora from variations of formulation and production conditions. The natural lactic acid bacteria *Lactobacillus plantarum* and *Lactobacillus brevis* dominated independent of temperature extremes, addition of starter culture or whey. The results correspond with knowledge from wine production where the natural yeast flora plays a role for the final quality despite any addition of wine yeasts as a starter (Hühn and Gafner 1997). Pure fermentations are only reached with prior pasteurization of the must (Hasselbeck 2000; Hühn and Gafner 1997). Nevertheless heat treatment of herbal extracts cannot be proposed as optimization measure because many of the valuable plant compounds are heat labile.

It is worth mentioning that *Lactobacillus rossiae*, just isolated and classified from sourdough in 2005 (Corsetti et al. 2005) and otherwise known as beer-spoilage bacteria (Back 2009), was detected in three *Atropa belladonna* extracts together with further unknown *Lactobacillus* species.

2.9. Conclusion

The aim of this work was to study the aqueous-fermentative extraction methods of the German Homoeopathic Pharmacopoeia, HAB 2003, regulation nos. 33 and 34 developed many years ago by empirical experience without any more recent scientific consideration. The extraction is accompanied by fermentation and the mother tincture resulting matures for at least 6 months. In this part of the study the biochemical reactions from aqueous-fermented extracts from the fresh flowering herb of *Atropa belladonna* var. *belladonna* (L.) were studied and the responsible microorganisms were investigated.

The results show that lactic acid bacteria (LAB) are primarily involved in fermentation, which mainly produce lactic acid besides acetic acid and ethanol. The homofermentative *Lactobacillus plantarum* and the heterofermentative *Lactobacillus brevis* were identified as predominant lactic acid bacteria with a dominance of *Lactobacillus plantarum* in the main fermentation period. The processes come to an end after 13 weeks at the latest and the lactate formed is responsible for extract stability.

Furthermore it was shown that yeasts may play a role as secondary cultures and may reduce the extract stability by metabolizing the lactate. The proliferation of the specified microorganisms *Pseudomonas aeruginosa*, *Staphylococcus aureus*, gram negative enterobacteria and moulds as well as the formation of endotoxins can be excluded under the production condition of the HAB. Moreover, it was proven that a formation of methanol by failed fermentation does not occur in the extracts and is exclusively due to hydrolysis of the plant pectin.

By varying different extraction conditions contrasting current practice of the HAB, the following factors for a successful fermentation can be stated: Addition of carbohydrates, usage of a plant genuine starter culture, manufacturing with early harvested and unwashed plant material, maceration at 37 °C, late time of pressing, air-less maceration and fermentation as well as a short storage period. In contrast to this, the addition of ash does not show any impact on extract quality in the case of *Atropa belladonna*.

In most cases, the evaluated manufacturing procedure yielded a stable extract whereas microflora and fermentation courses were similar despite the variations of manufacturing parameters. With the exception of the worst-case-trials, further spoiled batches are most probably attributed to the natural variability of the genuine microbial flora of the starting material. In this context, the following proposals to optimize the current method can be made: Inoculation with a starter culture, addition of whey, continuous maceration at 37 °C, flexible dosage of carbohydrates in dependence of buffer capacity and fermentation process, air-less treatment during the first week as well as a shorter individual maturation period. These recommendations are first valid for the plant studied *Atropa belladonna*, flowering herb. However, as the results of the present study are in line with investigations on other plant materials (sauerkraut, sourdough, silage), it can be assumed that the suggestions made will also increase the manufacturing safety and induce a reproducible quality by using other plants as well as by performing the production methods of the German Homoeopathic Pharmacopoeia: regulation no. 35–37 and 51 [HAB 2003, regulation no. 35–37, 51] related

Table 3: Variation of the formulation components as opposed to standard formulation

Component	Source	Variation as opposed to the standard formulation
Honey (Ph. Eur.)	WALA Heilmittel GmbH, Bad Boll/Eckwälden, Germany; Walter Lang Honigimport, Bremen, Germany; Demeter Imkerei Bernhard Henschke, Waldstetten, Germany	Different amounts from 0 to 2.5 parts
Lactose Monohydrate (Ph. Eur.)	Molkerei MEGGLE Wasserburg GmbH, Wasserburg, Germany	Different amounts from 0 to 2.5 parts
Whey (HAB)	Procedure of HAB (HAB 2008, H 5.3 Whey): Incubation of inoculated pasteurized milk (Choozit™ 102 LYO 50 DCU (0.02 g/l) and Choozit™ Hel C FRO 10 D (0.1 g/l), Danisco Deutschland GmbH, Niebüll, Germany) for 2 days at 28 °C, filtration	HAB procedure, regulation no. 34c (HAB 2003) Batch size: 160 g plant, PER = 1:1.7-1.8
Starter culture	*Lactobacillus plantarum* type I, isolated from *Atropa belladonna* extracts in 2007 (see above)	Addition of 3 ml (10^8 CFU/ml)
Ash	Own preparation (see above)	The content of a big maceration pot was split in 6 bottles with different amounts of ash from 0 to 75 mg/100 g. Regulation no. 33a: 2000 g plant, PER = 1:1.0 Regulation no. 34c: 1200 g plant, PER = 1:1.7
Ethanol 96 % (V/V, Ph. Eur.)	Brüggemann, Heilbronn, Germany	Extraction according to Ph. Eur. regulation no. 2a with the following modifications (Ph. Eur. 6th edition: Methods of preparation of homoeopathic stocks and potentisation, regulation no. 2a): • adaption of the ethanol amount to the water amount of the standard formulation • maceration for 3.5 d at RT, pressing • stirring in the morning and evening for 7 days • filtration and filling after 7 days • no addition of ash, honey or lactose • 400 g plant, PER = 1: 1.0-1.1

PER = plant extract ratio

with the ones studied. Therefore, the variants mentioned in this work should be tested on other plants as well as according to the related production methods to generalize the present results.

3. Experimental

3.1. Plant material

Fresh overground parts of *Atropa belladonna* L. var. *belladonna* (Solanaceae) were obtained from wild collections in Germany and were supplied by Heilkräuter Gorges (Buch, Germany; harvesting location: Boppard or Magstadt) or were harvested by Martina Schwarzenberger (harvesting location: Swabian Alb, Bad Boll). Between 2006 and 2009 16 batches were sourced for analysis and manufacture altogether. Harvest was performed at flowering time while the plant could bear little green berries. Plants bearing less blossoms, but big green to tender violet berries were separately processed to study the impact of a late point of harvest. For further processing the upper parts of the overground plant were chosen while the lower parts were discarded. Damaged and dead leaves as well as insects were removed.

3.2. Manufacture of the extracts

3.2.1. Standard manufacturing procedure of the aqueous-fermented extracts

The standard manufacturing procedure was based on the German Homoeopathic Pharmacopoeia HAB, regulation no. 33a as shown in Table 1 (HAB 2003, regulation no. 33a).
Comminution, maceration and pressing: After cleaning the plant material was comminuted by a cutter (K 90 AC 8, Seydelmann, Stuttgart, Germany). Honey and lactose were dissolved in purified water and the plant was added. The standard batch size amounted to 400 g plant. The pots were macerated in warming (37 °C) and cooling steps (ice-water) according to HAB. After some cycles the fermentation started: the pH fell down for at least 0.2 units to the first value, the odour went sour and bubbles appeared. Up to this point the pots were stored at room temperature during the warming phase. After 3.5 days the macerates were pressed through a cotton cloth during the cooling phase adhering to a plant extract ratio (PER) of 1:0.9–1.2. Further storage was carried out according to HAB for 3.5 days.
Filtration, ashing and filling: After filtration extracts were filled in bottles with rubber caps. The plant residue was air-dried or dried by a drying cabinet at 105 °C and was subsequently ashed in porcelain crucibles on Bunsen burner. The ash amount according to HAB (50 mg/100 ml) was changed to 50 mg/100 g because of reasons of practicability. The resulting mixture is the mother tincture according to HAB.
Storage and maturation: The extracts were stored protected from light at room temperature. Sterile cannulas were put into the rubber cap to allow fermentation gases to escape. The extracts were not moved with the exception of the sampling.

3.2.2. Variation of the standard manufacturing procedure

Table 3 and Table 4 show the variations of the general manufacturing procedure based on the standard formulation according to HAB, regulation no. 33. The respective test conditions are each recognizable in the name of the variants e.g. honey 2.5 means standard procedure with 2.5 parts honey. The comparative batches were made as series from the same herbal material to ensure constant conditions. Each variant was produced at least twice. Parallel batches of the standard formulation were produced to study reproducibility. For the same reason the extract of a big maceration pot (1400 g plant, PER 1:1.1–1.2) was split in several bottles to secure the same starting points und to study the further process. Between 2006 and 2009 106 aqueous-fermented and 4 ethanolic extracts were produced.

Table 4: Variation of the production conditions as opposed to the standard procedure

Production condition	Variation as opposed to the standard procedure
Time of harvest	Extracts of standard (honey 0.75), whey and ash series were prepared in the beginning and the end of the flowering time over several years
Washing the plant	Washing the plant before comminution, tested with standard and whey formulation
Maceration temperature during the first 7 days	Maceration at 37 °C ⇔ Ice, then RT ⇔ Ice (standard); 37 °C ⇔ Ice; RT ⇔ Ice; 37 °C; RT and 2-8 °C
Time of pressing	Pressing after 3 h, 12 h, 24 h, 36 h and 3.5 d (standard)
Oxygen access (negative control)	Two fivefold batches were produced where the cap went porous in the first weeks because of the fermentation gazes. The extracts spoiled and were analyzed to study the aerobic influence.
Maturation period	After filling samples were drawn after: • one or two days • 4 weeks • 12 weeks • 7 months • 12 months (extracts from 2006 and 2007)

3.2.3. Sampling

Samples were taken during manufacture and storage by sterile syringes and cannulas through the rubber cap. Extracts were shaken before sampling until the first day after filling. After this point the extracts were not moved any more. Samples were filled in sterile cryovials® (Simport, Beloeil, Canada), with the exception of the samples for endotoxin testing, for which special vessels were used (Cellstar®, Greiner Bio-One, Frickenhausen, Germany). Microbiological tests were carried out promptly after sampling whereas samples for analytical tests were frozen at least at −25 °C until analyses. In Table 5 sampling points and tests carried-out are compiled.

3.3. Analytical methods

3.3.1. Chemicals

MRS agar and bouillon for microbiology and acetone (p.a.) were obtained from Merck (Darmstadt, Germany). Acetaldehyde (for synthesis) was supplied from Merck Schuchardt (Hohenbrunn, Germany). Ethanol (99.2 %), ethyl acetate (99.9 %), methanol (99.8 %), 1-propanol (99.5 %) and 2-propanol (99.8 %) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Helium (p.a.) was obtained from MTI-Industriegase (Ulm, Germany). NaCl peptone buffer/0.1 % Tween 80 was supplied from heipha Dr. Müller (Eppelheim, Germany). Deionized water (0.055 µS/cm, 25 °C), hydrogen (p.a.) and synthetic air (p.a.) were generated in house.

3.3.2. pH

The pH was determined potentiometrically (Mettler Toledo, Giessen, Germany) twice a day (Ph. Eur. 6th edition: 2.2.3: Potentiometric determination of pH) during the first week and at the points listed in Table 5.

3.3.3. Galactose, lactose, D/L-lactate, starch

The substances were determined enzymatically after filtration (0.2 µm, Millex®-GN, Millipore, Billerica, Massachusetts, USA; Rotilabo®-Spritzenfilter, Roth, Karlsruhe, Germany) and dilution if necessary. The test was carried out as described in the information leaflet of the test kit (Boehringer-Mannheim/r-Biopharm, Darmstadt, Germany) determining the absorbance at a wavelength of 340 nm. The limits of quantification were 0.2 g/l for lactose or galactose and 0.03 g/l for D- or L-lactate and starch.

3.3.4. Glucose, fructose, sucrose, acetate

Fructose, glucose, sucrose and acetate were determined enzymatically by Jordananalytik (Eibelstadt, Germany) after filtration (0.2 µm, Millex®-GN, Millipore, Billerica, Massachusetts, USA; Rotilabo®-Spritzenfilter, Roth, Karlsruhe, Germany). The test was performed automatically by a Konelab 60 system (Thermo Scientific, Waltham, Massachusetts, USA) at a wavelength of 340 nm with enzymes and buffers from r-Biopharm (Darmstadt, Germany). The limit of quantification was 0.1 g/l for all substances.

3.3.5. Methanol, ethanol, 1-propanol, 2-propanol, ethyl acetate, acetaldehyde, acetone

Compound determination was performed by headspace GC based on Ph. Eur. 2.9.11 (Ph. Eur. 6th edition: 2.9.11: Test for methanol and 2-propanol) with 100 µl filtered sample (0.2 µm, Millex®-GN, Millipore, Billerica, Massachusetts, USA; Rotilabo®-Spritzenfilter, Roth, Karlsruhe, Germany). Equipment was from PerkinElmer (Waltham, Massachusetts, USA; headspace: Turbo Matrix HS-16; gas chromatograph, FID: Clarus 500) and a capillary column from Phenomenex (Torrance, California, USA; 14 %-cyanopropylphenyl 86 %-dimethylpolysiloxan 60 m x 0.25 mm x 0.25 mm, ZB-1701). Operation conditions were He: 130 kPa; FID: 250 °C; fuel gas: H_2 45 ml/min and synthetic air 450 ml/min; injection temperature: 180 °C; split: 30:1. Results were reported by TotalChrom 6.3.1 (PerkinElmer, Waltham, Massachusetts, USA). The following program was applied: 0–15 min, 40 °C; 15–18 min, 40 → 70 °C; 18–24 min, 70 → 250 °C; 24–25 min, 250 °C. Retention times were 4.9 min for acetaldehyde, 5.1 min for methanol, 5.8 min for ethanol, 6.1 min for acetone, 6.3 min for 2-propanol, 8.0 min for 1-propanol and 8.3 min for ethyl acetate, respectively. Acetone and acetaldehyde were identified by comparison with standard substances while the other substances were determined quantitatively by calibration curves.

3.4. Microbiological methods

3.4.1. Total viable aerobic count of bacteria, yeasts and moulds and test for specified microorganisms

Microbiological tests were performed by L&S (Bad Bocklet, Germany). The total viable aerobic counts of bacteria, yeasts and moulds were determined according to Ph. Eur. 2.6.12, method A (Ph. Eur. 6th edition: 2.6.12: Microbiological examination of non-sterile products: Total viable aerobic count). Specified microorganisms (absence of *Pseudomonas aeruginosa*, *Staphylococcus aureus,* Enterobacteria) were determined according to Ph. Eur. 2.6.13, method A (Ph. Eur. 6th edition: 2.6.13: Microbiological examination of non-sterile products: Test for specified micro-organisms). The test volume of both tests was reduced to 1.0 g.

3.4.2. Content of bacterial endotoxins

The test was performed by L&S (Bad Bocklet, Germany) according to Ph. Eur. 2.6.14, method C turbidimetric kinetic method (Ph. Eur. 6th edition: 2.6.14: Bacterial endotoxins). The extracts were directly analyzed. Plant material was suspended in *water for bacterial endotoxins test* (100 mg/ml), homogenized for 5 min, centrifuged for 5 min at 2000 rpm and the supernatant was analyzed.

3.4.3. Identification of yeasts

The identification of yeasts obtained according to 3.4.1 was performed biochemically by API systems (20 C AUX, 32C; bioMérieux, Marcy-l'Etoile, France) by L&S (Bad Bocklet, Germany). The three most countable, morphologically different yeasts of the extracts in 2007 were identified.

3.4.4. Isolation and identification of lactic acid bacteria

Isolation of the lactic acid bacteria from *Atropa belladonna* plant material was performed that 10 g plant material was homogenized with 90 ml NaCl peptone buffer/ 0.1 % Tween 80. The liquid obtained was filtered by EZ-Pak® membrane filter (Millipore, Billerica, Massachusetts, USA) and rinsed with 90 ml NaCl peptone buffer/0.1 % Tween 80. The filter was transferred into 90 ml MRS bouillon (52.2 g/l, pH 5.6) and incubated for 2 days at 29 °C.

Table 5: Sampling of extracts and tests carried out

Sample point	Analytical tests	Microbiological tests
Fresh plant/fresh plant juice	• Fermentation substrates: glucose, fructose, galactose, lactose, sucrose, starch • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids, flavonoids *	• Total viable aerobic count, specified microorganisms • Identification of lactic acid bacteria • Endotoxins
After pressing	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids, flavonoids *	• Total viable aerobic count, specified microorganisms
After 7 days (after filtration)	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, methanol, ethanol and other alcohols • Alkaloids *	/
After 8 or 9 days (1 day after filling)	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, methanol, ethanol and other alcohols • Alkaloids, flavonoids *	• Total viable aerobic count, specified microorganisms • Identification of lactic acid bacteria • Endotoxins
After 5 weeks (4 weeks after filling)	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids *	• Total viable aerobic count, specified microorganisms
After 13 weeks (12 weeks after filling)	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids *	• Total viable aerobic count, specified microorganisms
After 7 months	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids, flavonoids *	• Total viable aerobic count, specified microorganisms • Identification of lactic acid bacteria • Endotoxins • Identification of yeasts
After 12 months	• pH • Fermentation substrates: glucose, fructose, galactose, lactose, sucrose • Fermentation products: D/L-lactate, acetate, methanol, ethanol and other alcohols • Alkaloids *	• Total viable aerobic count, specified microorganisms

* Data will be presented in part 2 of the paper series.

The bouillon was spread on MRS agar (68.2 g/l, pH 5.6) and incubated for 2 days at 29 °C.
Isolation und determination of the microbial count of the lactic acid bacteria were carried out by L&S (Bad Bocklet, Germany) based on the methods of § 64 LFGB, method L06.00–35. The extract was homogenized 1:9 with a buffer (pH 7.0, NaCl (4.3 g/l), KH_2PO_4 (3.6 g/l), $Na_2HPO_4 \times 2\ H_2O$ (7.2 g/l), peptone (1.0 g/l), histidine (1.0 g/l), Tween 80 (10 ml/l), lecithin (3.0 g/l). 100 µl of the mixture were spread on MRS agar and incubated for 72 h at 25 °C. The three most countable, morphologically different LAB from the extracts in 2007 and 2008 were identified (see below).

10 g plant material was mixed with 90 ml buffer and the supernatant was spread on MRS agar.
The lactic acid bacteria were identified by the Department of Technical Microbiology of the Technical University Munich, Germany (Prof. Dr. Rudi F. Vogel).
According to previously published methods (Aznar and Chenoll 2006; Ehrmann et al. 2009; Harmsen 2005; Welsh and McClelland 1990) the DNA of the LAB was isolated, the 16SrRNA gene was amplified and identified to the half (800 to 900 bases). The comparison with a data base allowed the identification of the species. In some cases a type differentiation was performed by DNA fingerprints: RAPD patterns (random amplified polymorphic DNA) with primer M13 (5'-GTTTTCCCAGTCACGACGTTG-3') were used.

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