

# Monitoring Gram positive bacterial contamination in Czech breweries

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*Quality control procedures were monitored in a number of Czech breweries. All Gram positive bacterial contaminants found in the beer were collected and isolated. The occurrence of this contamination was recorded.*

*Gram positive bacteria obtained from the breweries were isolated and identified by API 50 CHL. This method is generally used as a comparative method for identification of lactic acid bacteria.*

*Pasteurised beer was inoculated with all identified bacteria in the absence of oxygen. Haze formation was checked over three months. The results show that different beer spoilage was observed on inoculating beer with isolates identified as identical.*

**Key Words:** *Beer spoilage, lactic acid bacteria, isolation and identification.*

## INTRODUCTION

Contaminating micro-organisms constitute a significant risk to all types of food producers including the brewing industry. Microorganisms considered cultural for one food commodity can become a biological disaster in another food branch. For instance, lactic acid bacteria that form the cultural microflora in the dairy industry would be highly undesirable in other food lines. In the brewing industry, these bacteria may cause unpleasant sensory changes in beer and occurrence in high numbers may cause the complete destruction of the finished product.

When compared with other food industries, the brewing industry has a considerable advantage. Its final product is a poor substrate for many micro-organism groups due to its low pH and content of nutritive substances the high ethanol content and degree of CO<sub>2</sub> saturation and the presence of hop bitter substances<sup>5,11</sup>. Pathogenic micro-organisms, such as salmonellae and listeria, do not reproduce or survive in beer.

These advantages do not apply to the whole brewing process. Thus during the slow cooling of hopped wort, which is an ideal substrate for microflora development, some groups of micro-organisms may proliferate so quickly that they may cause irreversible sensory changes before their growth is checked by the fast growth of cultural yeast during the primary fermentation<sup>1,10</sup>.

Finished beer may also contain some micro-organisms able to survive and reproduce even under unfavourable conditions. These are mostly certain species of strict and facultative anaerobes – wild yeast or Gram positive and

catalase negative lactic acid bacteria<sup>5,11</sup>. These micro-organisms, though not representing any health risk, are a problem for quality control<sup>12</sup>. For quality control in a brewery, even cultural brewery yeast must be considered a contaminating micro-organism if found in beer after the filter.

Nearly every yeast species present in finished beer may produce sediment within 7 days, lactic acid bacteria can do so within 1 month. Due to the current trend of extending shelf-life, the possible occurrence of yeast in the finished product may cause considerable spoilage. Each food company therefore pays close attention to control by means of sanitary precautions and cleanliness of the production equipment.

The present state of plant laboratory equipment makes it possible to obtain results of chemical analyses for the product and semiproduct control within one hour of sampling. This time limit, of course, is not valid for biological control. To assay a sample for the presence of yeast using the classical plating methods and counting the resulting colonies takes up to 48 hours. For Gram positive lactic acid bacteria, which represent the greatest hazard to the quality of the finished beer, the delay from sampling to counting the colonies is even longer (5 to 7 days) depending on the microbial species<sup>6</sup>. This is why plant microbiologists keep searching for faster methods of biological control.

The literature describes a considerable number of so-called rapid methods for the assay and identification of micro-organisms<sup>4,11,13,14</sup>, but not all of these are suitable for plant laboratory conditions. To verify some of those

methods, we investigated the occurrence of contaminating micro-organisms, primarily Gram positive bacteria, in Czech breweries. The contaminants were then isolated, identified and stored to form a collection.

## MATERIALS AND METHODS

### Media

Sterile low-fat milk was used for the conservation of frozen isolates of lactobacilli and pediococci<sup>8</sup>.

MRS agar (Oxoid) with addition of actidione (0,025 g/litre) and  $\beta$ -phenylethanol (0,3%) was used for isolation and conservation of Gram positive and catalase negative of lactobacilli and pediococci<sup>6</sup>.

MRS broth (Oxoid) was used for propagation of isolates of lactobacilli and pediococci.

MRS agar (Oxoid) was used for propagation of isolates of lactobacilli and pediococci prior to identification.

### Materials

The McFarland turbidity scale was used for estimation of bacterial concentration prior to identification<sup>2</sup>.

API CHL sets used for identification of lactic acid bacteria were produced by BioMérieux.

180 ml vials with safety closures were produced by Barby and Kuhner, Untersiemau, BRD.

Membrane filters, (Millipore, pore size 0,45  $\mu$ m) were used for sample concentration.

### Operation procedure

To acquire contaminant isolates, samples were taken from young beers before racking, the fermenting cellar in plants with classical fermentation, beers before filtration, after filtration, before pasteurisation and finished beers.

Individual micro-organisms were isolated by sample concentration using membrane filtration and subsequent cultivation on solid selective media at 28-30°C. Individual resulting colonies grown from a single cell were isolated by streaking. This procedure was repeated several times and the acquired isolates were then conserved for identification in the respective media. The isolated micro-organisms were cultivated in selective broths. Lactic acid bacteria, preserved on MRS broth at +4 to +6°C, were subcultured once in 14 days, those placed in sterile low-fat milk at of -20°C once in 6 months.

Lactic acid bacteria were identified using API 50 CHL kits for identification of lactobacilli and pediococci. Based on biochemical tests on 49 carbohydrate media

located in plastic microtubes, the procedure permitted the identification of 52 strains of lactic acid bacteria. Prior to identification, these bacteria were reproduced to obtain the necessary cell count. Simultaneously with the seeding of the identification kits, 10% beer from a sampling site after the brewery flow pasteuriser was also inoculated (sampled) in a sterile manner. This was done to assess the ability of individual isolates to spoil pasteurised beer. The vials were filled with pasteurised beer up to the rim in order to exclude oxygen.

Lactic acid bacteria isolates were propagated on respective solid broths inoculated by streaking. After 72-120 hours of cultivation, the cells were resuspended with an inoculating loop into a small volume of sterile physiological saline. With simultaneous counting of drops, the suspension was diluted so that its turbidity corresponded to the No.2 standard of the McFarland scale. A double number of drops from the original thick saline was then added into the inoculating medium containing a colour indicator, and thoroughly mixed. Bubble formation was prevented. Individual API 50 CHL microtubes were then filled with this inoculating medium by micropipettes. After inoculation, sterile paraffin oil was dropped into the wells over the tubes in order to secure anaerobic conditions and the whole set was covered by a lid and cultivated in a thermostat at 30°C.

The resulting microbial suspensions were further diluted to correspond to McFarland No. 1 turbidity, i.e.  $300 \times 10^6$  bacteria/ml. One drop of this suspension was used to inoculate a vial with a safety closure (filled to the rim to ensure anaerobic conditions) with 10 % pale beer collected in the brewery from a sampling site after the flow pasteuriser. This quantity of cells corresponded to an inoculum of approximately  $5.5 \times 10^4$  bacteria/ml. The vials with inoculated beer were kept in the dark at room temperature and controlled twice a week over three months. The majority of beer-spoilage isolates caused massive turbidity in beer within 3 weeks at the latest; at later dates, the turbidity was observed only exceptionally. The time limits for deposition were 3 and 8 weeks since no further spoilage was observed at longer time intervals (Tables I and II).

## RESULTS AND DISCUSSION

Samples were collected from different production stages beginning with young beers and ending with finished unpasteurised beers. The samples were collected from 16 Czech breweries, each with an annual shipped beer volume of at least 10 000 hl. Samples from two breweries did not contain any contaminating micro-organisms. From the biological point of view, the majority of the other breweries had effective quality procedures, only 25 isolates of yeast contaminants and 63 isolates of Gram positive bacteria being intercepted during the 4-year period. No strictly anaerobic

TABLE I. Identification of isolates of lactic acid bacteria found in Czech breweries.

Number of isolate	Identification of isolates	Image under microscope	Beer spoilage		Brewery
			in 3 weeks	in 8 weeks	
1	<i>L. plantarum</i>	mr - lr, ch	-	-	N
2	<i>L. plantarum</i>	mr, ch	-	+	N
3	<i>L. para. paracasei</i>	mr, ch	-	+	N
4	<i>L. brevis</i>	lr - vlr	-	-	N
5	<i>L. collinoides</i>	mr - lr, ch	+	+	N
6	<i>L. plantarum</i>	mr - lr, ch	+	+	N
7	<i>L. buchneri</i>	sr - mr	-	-	N
8	<i>L. buchneri</i>	sr - mr	+	+	N
9	<i>L. plantarum</i>	sr - lr	+	+	N
10	<i>L. plantarum</i>	mr	-	-	N
11	<i>L. brevis</i>	lr	-	-	N
12	<i>L. brevis</i>	lr	-	-	N
13	<i>L. brevis</i>	mr	-	-	N
14	<i>L. brevis</i>	mr	-	-	N
15	<i>L. brevis</i>	mr - lr	-	-	N
16	<i>L. fructivorans</i>	lr, in pairs	-	-	N
17	<i>L. collinoides</i>	lr	-	-	N
18	<i>L. brevis</i>	lr	+	+	D
19	<i>L. brevis</i>	lr, ch	+	+	D
20	<i>L. buchneri</i>	sr, in pairs	-	-	D
21	<i>L. buchneri</i>	sr - mr, in pairs	-	-	I
22	<i>L. brevis</i>	lr	+	+	I
23	<i>L. plantarum</i>	mr - lr	-	+	I
24	<i>L. buchneri</i>	mr	-	+	I
25	<i>L. plantarum</i>	sr - mr	-	+	K
26	<i>L. collinoides</i>	lr	-	-	G
27	<i>L. collinoides</i>	vlr	+	+	G
28	<i>L. brevis</i>	vlr	-	-	G
29	<i>L. brevis</i>	vlr	+	+	G
30	<i>L. plantarum</i>	vlr	-	-	G
31	<i>L. brevis</i>	vlr	+	+	G
32	<i>L. brevis</i>	vlr	-	-	G
33	<i>L. brevis</i>	vlr	-	-	G
34	<i>L. collinoides</i>	lr	+	+	N
35	<i>L. collinoides</i>	lr	-	-	N
36	<i>L. collinoides</i>	mr - lr	+	+	N
37	<i>L. brevis</i>	lr	+	+	N
38	<i>Leuc. mesenteroides</i>	lr	-	-	N
39	<i>L. brevis</i>	vlr	+	+	N
40	<i>L. brevis</i>	vlr	-	-	N
41	<i>L. plantarum</i>	vlr	-	-	N
42	<i>L. brevis</i>	mr - lr	+	+	N
43	<i>L. para. paracasei</i>	mr - lr	-	-	H
44	<i>Ped. damnosus</i>	cocci, tetrads	+	+	H
45	<i>L. para. paracasei</i>	mr, ch	-	-	H
46	<i>L. plantarum</i>	mr - lr	-	-	E
47	<i>L. brevis</i>	sr - mr, in pairs	-	-	E
48	<i>L. para. paracasei</i>	mr	-	-	E
49	<i>L. plantarum</i>	mr	-	-	E
50	<i>L. brevis</i>	vlr	-	-	F
51	<i>L. plantarum</i>	mr	-	+	B
52	<i>L. brevis</i>	lr	+	+	C
53	<i>Ped. sp.</i>	cocci, tetrads	-	-	J
54	<i>Ped. sp.</i>	cocci, tetrads	-	-	J
55	<i>L. brevis</i>	vlr	+	+	M
56	<i>L. brevis</i>	vlr	+	+	L
57	<i>Ped. sp.</i>	cocci, tetrads	+	+	L
58	<i>Ped. sp.</i>	cocci, tetrads	-	-	L
59	<i>Ped. sp.</i>	cocci, tetrads	-	-	L
60	<i>Ped. sp.</i>	cocci, tetrads	-	-	L
61	<i>L. brevis</i>	mr	-	-	L
62	<i>L. brevis</i>	sr, ch	-	-	A
63	<i>L. plantarum</i>	mr	+	+	A
CS	<i>L. plantarum</i>	mr	-	-	collection

CS control strain from dairy culture collection "Laktoflora", Prague

sr short rods in 2 nm      mr middle rods 2-4 nm      lr long rods 4-6 nm  
 vlr very long rods more of 6 nm      ch chain-forming rods

TABLE II. Frequency of isolation of Gram positive bacteria.

	Number of isolates			
	A	B	C	D
<i>L. brevis</i>	11	11	14	25
<i>L. plantarum</i>	3	7	6	14
<i>L. collinoides</i>	4	4	3	7
<i>L. buchneri</i>	1	2	3	5
<i>L. para. paracasei</i>	0	1	3	4
<i>L. fructivorans</i>	0	0	1	1
<i>Leuc. mesenteroides</i>	0	0	1	1
<i>Pediococcus sp.</i>	1	1	5	6
<i>Ped. damnosus</i>	1	1	0	1

A Isolates of bacteria from spoiled beer after 3 weeks.

B Isolates of bacteria from spoiled beer after 8 weeks.

C Isolates which did not spoil beer.

D Total number of isolates.

*Megasphaera* or *Pectinatus*<sup>7</sup> were found. The isolated micro-organisms were identified and conserved for future research.

The results of the identification of lactic acid bacteria isolates are shown in Table I. The collection of contaminant isolates from brewing plants has been preserved and the ability of contaminants to grow and reproduce in pasteurised beer is repeatedly verified. Further strains have been isolated and identified for research purposes. Bacterial contaminants included the genera *Lactobacillus* and *Pediococcus*, with a single isolate of *Leuconostoc*. The most frequent species were *L. brevis* (25x), *L. plantarum* (14x), *L. collinoides* (7x), *L. buchneri* (5x) and *L. para. paracasei* (4x), *L. fructivorans*, *P. damnosus* and *Leuc. mesen. mesenteroides* (1x). *Pediococcus* ssp. was identified 6 times, without further specification of species. Although the number of bacterial isolates was not very high, the most frequent occurrence of *L. brevis* and *L. plantarum* in finished beers from different breweries corresponds to the published data<sup>3,9</sup>.

The beer-spoilage ability of lactic acid bacteria isolates was found 11 times with *L. brevis*, 7 times *L. plantarum*, twice with *L. collinoides* and once with *L. buchneri*, *L. para. paracasei* and *P. damnosus*. Of the six isolates identified as *Pediococcus* ssp. only one strain repeatedly spoiled beer. The frequency comparison of beer-spoilage bacteria and non-beer-spoilage bacteria is shown in Table II. The different affinity of individual isolated varieties to beer as a substrate seems to be very promising for future research.

## CONCLUSION

A 4-year study of occurrence of contamination in Czech breweries indicated good level of sanitation in these plants. Hundreds of samples were taken from the

plants in order to acquire a sufficient number of isolates of Gram positive lactic acid bacteria. Most of the samples failed to show any growth of colonies on specific media.

During identification, some of the lactic bacteria isolates exhibited small deviations from biochemical reactions typical for the given strain. These deviations did not preclude an exact classification of the micro-organism but pointed to the very probable formation of new varieties resulting from long-term survival under atypical external conditions. This corresponded also with the ability of the bacteria to utilise beer as the sole substrate for growth and reproduction.

The results of this monitoring study form a basis for further research, which could permit a fast differentiation between different microorganisms and facile determination of their ability to survive and reproduce under the unfavourable conditions they encounter in beer as a nutrient medium.

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