



Microbiology of brewing production - bacteria of the order Enterobacterales and culture methods for their detection

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Abstract

The growth of 7 strains belonging to the order of *Enterobacterales*, represented by the species of *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *Obesumbacterium proteus*, *Rahnella aquatilis*, *Raoultella terrigena*, *Serratia marcescens* and *Shimwellia pseudoproteus*, was monitored on selected cultivation media. Three types of agars - Endo, MacConkey and Chromocult Coliform agar together with two incubation temperatures of 28 and 37 °C were tested under aerobic conditions. The aim of the study was to detect such essential enterobacteria harmful to beer that cannot be proven at 37 °C, which is the temperature usually used in operational laboratories in breweries. Our results showed that most of the tested strains of enterobacteria were able to grow at 28 °C on all selected types of agar. The exception was just the representatives detection of which is problematic at 37 °C. Nevertheless, a little or no growth was always observed on just one of the tested media.

Keys words: enterobacteria, facultative-anaerobic bacteria, *Klebsiella*, coliform bacteria, wort contamination, *Obesumbacterium*, *N*-nitrosamines, *Rahnella*, *Serratia*, *Shimwellia*

1 Introduction

This article follows a review paper entitled Microbiology of brewery production – bacteria of the order of *Enterobacterales* (Matoulková et al., 2018). The *Enterobacterales* order contains 7 families with more than 40 bacterial genera that are isolated from diverse types of environments. Particularly members of *Shimwellia*, *Obesumbacterium*, *Rahnella*, *Citrobacter*, *Klebsiella*, *Raoultella*, *Serratia* and *Enterobacter* genera were found in breweries. On the other hand, pathogenic species, including *Escherichia coli*, have not been detected in breweries (Van Vuuren and Priest, 2003).

Mainly due to the sensitivity of enterobacteria to ethanol and acidic pH, their growth and ability to multiply in the finished beer is minimal and they occur mainly as a contamination of production yeast.

Contaminated water or unsatisfactory hygienic condition of surfaces and equipment (e.g. leakage of pipe

connection) represent other usual sources of enterobacteria (Vaughan et al., 2005). These bacteria are harmful since they produce undesirable sensory substances (e.g. diacetyl and dimethyl sulphide) at the beginning of the main fermentation. Moreover, some species are involved in the formation of carcinogenic *N*-nitrosamines (Boulton and Quain, 2001).

Shimwellia pseudoproteus and *Rahnella aquatilis* present an increased risk as they can repeatedly penetrate into a new batch of wort as contaminants of pitching yeast and therefore they are able to damage several batches of beer in a row (Bokulich and Bamforth, 2013). The damaged beer then shows a sweet, honey, fruity to vegetable and even fecal character (Jespersen and Jakobsen, 1996; Briggs et al., 2004).

The detection of enterobacteria in operational brewing laboratories is a routine procedure that is carried out mostly on MacConkey agar or chromogenic media at 37 °C.

In this study, we compared the growth of selected species of enterobacteria on several types of cultivation agars - Endo, MacConkey and Chromocult Coliform agar at 28 °C (the temperature recommended for incubation of some *Shimwellia*, *Obesumbacterium* and *Rahnella* species) and 37 °C (the temperature recommended for incubation of some *Citrobacter*, *Klebsiella*, *Raoultella* and *Serratia*).

2 Material and Methods

2.1 Cultivation media

- **Plate Count agar (PCA):** 22.5 g of PCA powder (Merck) was dissolved in 1 000 mL of distilled water and sterilized at 121 °C for 20 minutes.
- **Nutrient agar (NA):** 20 g of NA powder (Merck) was dissolved in 1 000 mL of distilled water and sterilized at 121 °C for 20 minutes.
- **Endo agar:** 39 g of Endo agar powder (Merck) was dissolved in 1 000 mL of distilled water and sterilized at 121 °C for 20 minutes.
- **MacConkey agar:** 50 g of MacConkey agar powder (Merck) was dissolved in 1000 mL of distilled water and sterilized at 121 °C for 20 minutes.
- **Chromocult Coliform agar:** 26.5 g of Coliform agar powder (Merck) as dissolved in 1 000 mL of distilled water and sterilized at 121 °C for 20 minutes.

2.2 Microorganisms and cultivation conditions

The used bacterial strains came from the following col-

lections: Czech Collection of Microorganisms (CCM), the Collection of Brewing Microorganisms (RIBM) and the German Collection of Microorganisms and Cell Cultures (DSMZ). The list of strains, their marks and origins is given in Table 1. The strains were incubated under aerobic conditions on PCA agar at 28 °C for 48 hours before inoculation on experimental media.

2.3 Preparation of bacterial suspension and cultivation conditions

Bacterial suspensions were prepared by stirring 1 colony obtained from agar plate into a sterile physiological solution. The concentration of resulting cell was approximately 3×10^8 cells/mL. The suspensions were diluted in order to grow a countable number of colonies on Petri dishes (i.e. 10–50). Their incubation was maintained under aerobic conditions at 28 and 37 °C for 48 hours.

3 Results and discussion

All bacterial strains grew on basic culture media (NA and PCA) and showed no differences in the appearance of their colonies. This is demonstrated in Figure 1., where the colonies of *C. freundii* CCM 7187 on NA are present. Regular, flat to slightly convex smooth colonies with a glossy surface and white to slightly cream colour were formed. A similar description of the colonies is reported by such authors as Holt et al. (1994), Back (2005) or Cosmas et al. (2016).

Table 1 The list of tested strains, their designation and origin

Bacterial species	Strain*	Origin	Optimal growth temperature
<i>Citrobacter freundii</i>	CCM 4475	Frozen carrot	37 °C
	CCM 7187	Clinical material	37 °C
<i>Enterobacter cloacae</i>	CCM 7931	Brewery operation - water	30 °C
<i>Escherichia coli</i>	CCM 7395	Food sample	30 °C
	RIBM CH1	Brewery operation - water	30 °C
<i>Klebsiella oxytoca</i>	CCM 3565	Drinking water	37 °C
<i>Obesumbacterium proteus</i>	CCM 2806	Brewer's top fermenting yeast	30 °C
	DSM 2777	Brewer's top fermenting yeast	30 °C
<i>Rahnella aquatilis</i>	CCM 4086	Salt	30 °C
<i>Raoultella terrigena</i>	CCM 3568	Drinking water	37 °C
<i>Serratia marcescens</i>	CCM 303 ^T	Water	37 °C
	CCM 8587	Karst water	30 °C
<i>Shimwellia pseudoproteus</i>	DSM 3038 ^T	Brewer's top fermenting yeast	30 °C
	DSM 22121	Brewer's top fermenting yeast	30 °C

* CCM – Czech Collection of Microorganisms, Masaryk University in Brno, Czech Republic; DSM – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; RIBM – Collection of Brewing Microorganisms, Research Institute of Brewing and Malting, Czech Republic.

3.1 The growth of selected enterobacteria on Endo agar

Because of the toxicity and low stability of fuchsin, Endo agar is used only to a limited extent for detection of coliform bacteria. The selectivity of Endo agar is determined by the content of basic fuchsin (decolorized with sulphite), with the carbon substrate being lactose. The colour of colonies makes it possible to distinguish between lactose-positive (pink or red colonies possibly with a greenish metallic lustre) and lactose-negative (colourless and cream colonies) bacteria. In our case, pink and red colonies with a metallic lustre or without any lustre were observed on Endo agar (Figure 2).

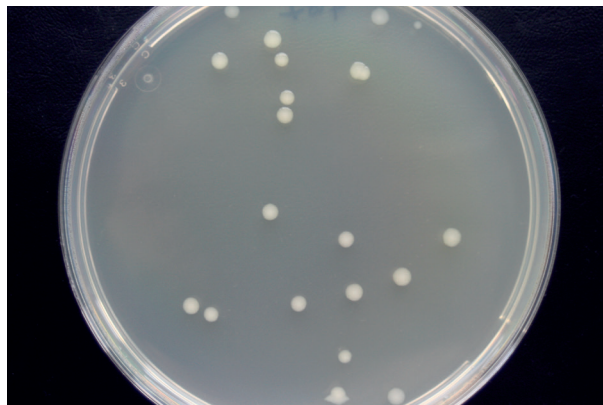


Figure 1 Morphology of *C. freundii* CCM 7187 colonies on nutrient agar

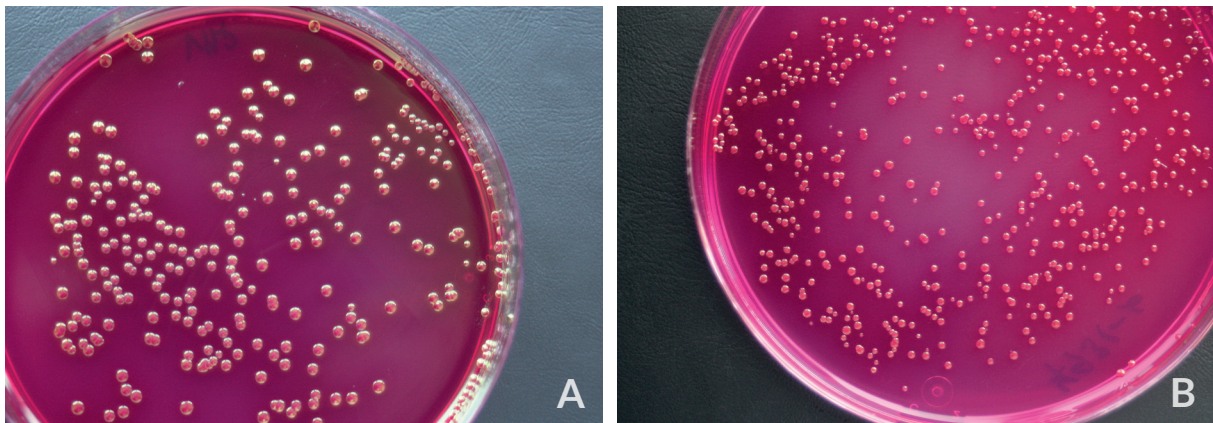


Figure 2 Morphology of enterobacteria colonies on Endo agar
A – *E. cloacae* CCM 7931 B – *E. coli* RIBM CH1

Colonies of *C. freundii* CCM 4475, *E. coli* RIBM CH1 and *K. oxytoca* CCM 3565 were red with a metallic lustre. Red colonies without a metallic lustre belonged to the strains of *E. coli* CCM 7395, *Enterobacter cloacae* CCM 7931, *R. terrigena* CCM 3568 and *S. pseudoproteus* DSM 22121. Various sources refer to a description of *E. coli* colonies: for instance Niemi et al. (2001) described the growth of *E. coli* as red colonies with a metallic lustre, while Back (2005) reported glossy and pink-red colonies.

Both strains of *O. proteus* (CCM 2806 and DSM 2777) as well as the strain of *S. marcescens* CCM 8587 provided pale pink colonies. Other bacteria grew in the form of pink colonies. *S. pseudoproteus* DSM 3038^T formed red metallic colonies on Endo agar. Endo agar was the only test medium on which the potentially risky bacteria of *S. pseudoproteus* DSM 3038^T and *R. aquatilis* CCM 4086 were growing at 37 °C. The presence of enterobacterial colonies on Endo agar is represented by *E. cloacae* CCM 7931 (Figure 2A) and *E. coli* RIBM CH1 (Figure 2B).

3.2 The growth of selected enterobacteria on MacConkey agar

Endo agar was gradually replaced by MacConkey medium especially due to the toxicity of fuchsin and because of numerous aspects from the field of clinical microbiology. MacConkey agar contains lactose as a carbon source and a neutral red as a pH indicator. The medium enables detection of lactose-positive (coliform) bacteria, which grow on agar plates in the form of pink colonies without changing the colour of the culture medium. Lactose-negative bacteria (e.g. *Obesumbacterium*, *Shimwellia*) form cream-coloured colonies on MacConkey agar and they discolour the medium into light yellow to ochre. Gram-positive bacteria are inhibited by crystal violet and bile salts (Finney et al., 2003). Bacterial growth on MacConkey Agar is documented in Figure 3.

Lactose-positive representatives of *C. freundii* CCM 4475, *E. cloacae* CCM 7931, *E. coli* CCM 7395 (Figure 3A), *K. oxytoca* CCM 3565 (Figure 3B) and *S. marcescens* CCM 303^T (Figure 3D) formed pink colonies without changing the colour of the medium at both incubation

temperatures. An exception was the strain of *R. aquatilis* CCM 4086, which did not grow at 37 °C at all. This finding corresponds to the basic characteristics of the genus – the temperature optimum for most representatives is between 25–35 °C; at 37 °C they grow significantly slowly (Kämpfer, 2015).

Lactose-negative bacteria of *O. proteus* DSM 2777 and *S. pseudoproteus* DSM 3038^T, formed cream coloured colonies at 28 °C and the medium is discoloured into light yellow to ochre as a result of their metabolism (Figure 3C). They formed no or only very poorly observable colonies at 37 °C.

The temperature optimum for most representatives is between 25 and 32 °C. 37 °C is the temperature commonly used for incubation of enterobacteria in brewing laboratories. It should be noted that the bacteria grow substantially more slowly at this temperature (Sedláček, 2007). Thus, our results show a risk that detection of harmful bacteria belonging to the genera of *Rahnella* or *Shimwellia* in beer will fail during cultivation on MacConkey agar at the recommended temperature of 37 °C.

3.3 The growth of selected enterobacteria on Chromocult Coliform agar

Chromocult Coliform agar was developed so that it could detect the total number of coliform bacteria together with the number of *E. coli* in one sample simultaneously. The medium contains two chromogenic substrates: Salmon-GAL to detect β-D-galactosidase (an enzyme that breaks down lactose into galactose and glucose) and X-glucuronide to detect presence of β-D-glucuronidase enzyme. Coliform bacteria show β-D-galactosidase activity. Although *E. coli* belongs to coliform bacteria, it can be distinguished from other coliform bacteria in the same agar plate due to β-D-glucuronidase activity. The growth of Gram-positive bacteria/organisms is inhibited by tergitol-7. Coliform bacteria grow on Chromocult Coliform agar in the form of pink to red colonies, while *E. coli* forms dark blue to purple colonies. The accompanying microflora grows in the form of colourless colonies (Finney et al., 2003). In our study

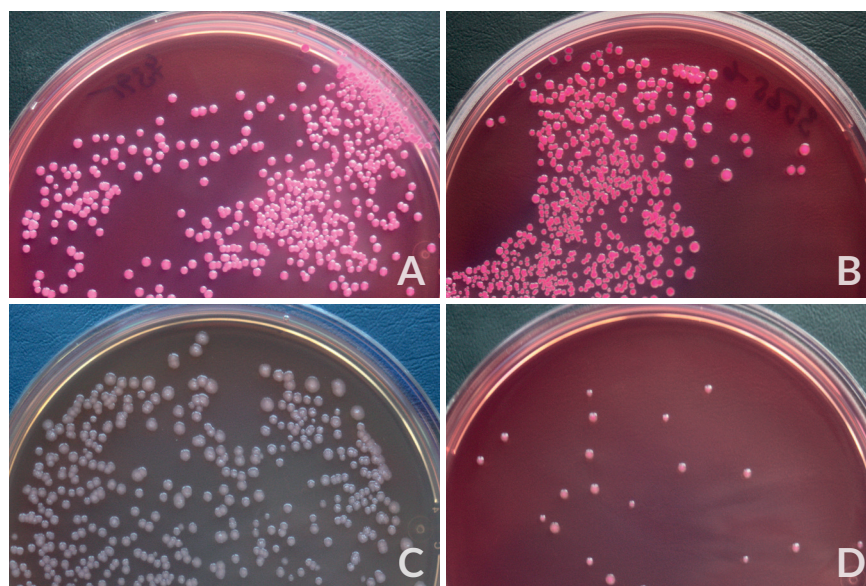
the formation of pinkish or light pink colonies was observed in cases of *Serratia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Rahnella* and *Shimwellia*. Detailed description for *E. cloacae* CCM 7931 is given in Figure 4A, for *K. oxytoca* CCM 3565 in Figure 4C. *O. proteus* strains DSM 2777 are elaborated in Figure 4D. *S. pseudoproteus* DSM 22121 grew as pink-purple colonies and both *E. coli* strains formed dark blue-violet colonies represented by *E. coli* strain RIBM CH1 shown in Figure 4B. Pink staining of *E. cloacae* and colonies of *Klebsiella* members, as well as blue-violet colour in *E. coli* have also been reported for example by Niemi et al. (2001).

3.4 The effect of incubation temperature on selected enterobacteria growth

The results of the growth of enterobacteria on various culture media at 28 and 37 °C are presented in Table 2. As for the temperature of 28 °C almost all tested bacterial strains grew on all types of media during this temperature. There are only two exceptions: the first being the strain of *O. proteus* CCM 2806 which generally grew more slowly and it did not grow at all on MacConkey and Chromocult Coliform agar soils. The other is *S. pseudoproteus* DSM 22121 which also did not grow on MacConkey agar. If we refer to the temperature of 37 °C, the strain of *O. proteus* CCM 2806 did not grow on any of the used medium, *R. aquatilis* CCM 4086 as well as *S. pseudoproteus* DSM 3038^T formed colonies only on Endo agar, *R. aquatilis* grew in no other culture media apart from Endo agar and *Shimwellia* did not grow at all or very weakly in any presented media.

Figure 3 Morphology of enterobacteria colonies on MacConkey agar

A – *E. coli* CCM 7395 B – *K. oxytoca* CCM 3565
C – *O. proteus* DSM 2777 D – *S. marcescens* CCM 303^T



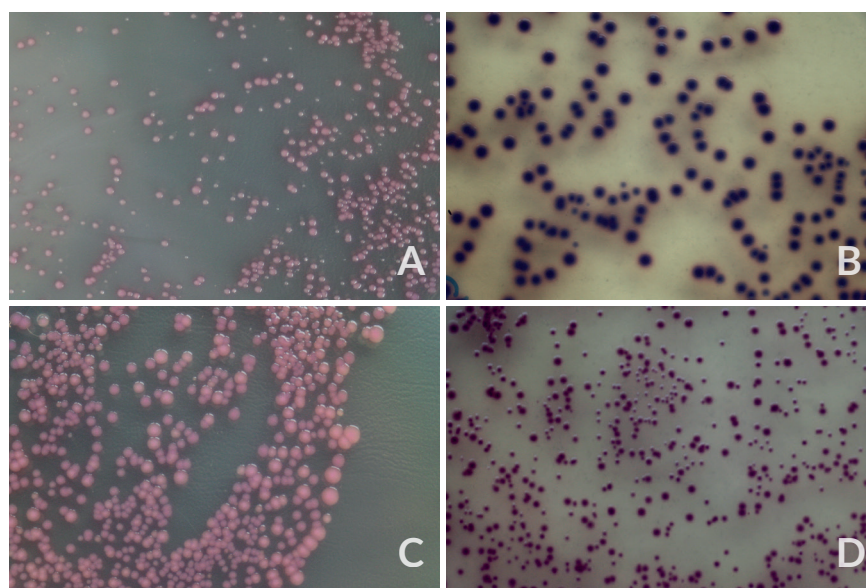
The presented results are consistent with the basic characteristics of the species. The temperature optimum of most members of the genera *Obesumbacterium* and *Shimwellia* is in the range of 25–32 °C, in the genus *Rahnella* 25–35 °C. At 37 °C, these bacteria grow significantly more slowly (Sedláček, 2007). The cultivation of enterobacteria in operational brewing laboratories takes place as standard at the temperature of 37 °C (Analytica EBC, 2011). Thus, the most risky taxa, such as *S. pseudoproteus* (“*O. proteus*”) and *R. aquatilis*, may not be detected during routine microbiological checks.

4 Conclusion

The presence of enterobacteria in samples that were taken in several breweries indicates deteriorating hygienic conditions and low levels of sanitation during the operation. Detection of enterobacteria is usually performed routinely in operational brewing laboratories. For diagnosis of en-

Figure 4 Morphology of enterobacteria colonies on Chromocult Coliform agar

A – *E. cloacae* CCM 7931 B – *E. coli* RIBM CH1
C – *K. oxytoca* CCM 3565 D – *O. proteus* DSM 2777



terobacteria, usually MacConkey agar or chromogenic media are used and the cultivation is carried at 37 °C. In our study we compared cultivations on different media – Endo agar, MacConkey agar, and Chromocult Coliform agar at two incubation temperatures of 28 °C and the commonly used 37 °C in order to monitor the growth character of enterobacteria. We proved that the cultivation at the above-mentioned 37 °C led to a slower growth or no growth at all in the case of the strains *Shimwellia pseudoproteus* (“*O. proteus*”)

Table 2 An overview of the enterobacteria growth at 28 and 37 °C

Species	Strain	Culture medium 28 °C / 37 °C				
		NA	PCA	Coli	Endo	Mac
<i>Citrobacter freundii</i>	CCM 4475	+ / +	+ / +	+ / +	+ / +	+ / +
	CCM 7187	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Enterobacter cloacae</i>	CCM 7931	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Escherichia coli</i>	CCM 7395	+ / +	+ / +	+ / +	+ / +	+ / +
	RIBM CH1	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Klebsiella oxytoca</i>	CCM 3565	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Obesumbacterium proteus</i>	CCM 2806	+ / -	+ / -	- / -	+ / -	- / -
	DSM 2777	+ / +	+ / +	+ / +	~ / ~	+ / ~
<i>Rahnella aquatilis</i>	CCM 4086	+ / -	+ / -	+ / -	+ / +	+ / -
<i>Raoultella terrigena</i>	CCM 3568	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Serratia marcescens</i>	CCM 303 ^T	+ / +	+ / +	+ / +	+ / +	+ / +
	CCM 8587	+ / +	+ / +	+ / +	+ / +	+ / +
<i>Shimwellia pseudoproteus</i>	DSM 3038 ^T	+ / ~	+ / -	+ / -	+ / +	+ / ~
	DSM 22121	+ / ~	+ / ~	+ / -	+ / ~	- / ~

+ standard growth; - no growth; ~ weak growth; NA, Nutrient agar; PCA, Plate Count agar; Coli, Chromocult Coliform agar; Endo, Endo agar; Mac, MacConkey agar

and *Rahnella aquatilis* (i.e. the taxa with the greatest potential of harmfulness from the point of view of microbiological control in a brewery). Thus, in laboratories that use only the incubation temperature of 37 °C for the detection of enterobacteria, some harmful strains may not be detected.

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