Formation of guaiacol in chocolate milk by the psychrotrophic bacterium *Rahnella aquatilis*

N. Jensen, P. Varelis and F.B. Whitfield  
*Food Science Australia, PO Box 52, North Ryde, NSW 1670, Australia*

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**Aims:** The aim of this study was to identify the causative agent of a smoky/phenolic taint in refrigerated full cream chocolate milk.

**Methods and Results:** Microbiological examination of spoiled and unspoiled milk samples from the same processor showed high numbers of the psychrotrophic coliform *Rahnella aquatilis* in the spoiled samples only. Gas chromatography/mass spectrometry (GC/MS) was used to identify and quantify the taint compound as guaiacol (2-methoxyphenol) in the spoiled milk. Challenge studies in UHT chocolate and white milks inoculated with the isolate and incubated at 4–5°C and 8–9°C for 6 d showed the production of guaiacol in chocolate milk only, which was confirmed and quantified by GC/MS.

**Conclusions:** The results indicate that if present in refrigerated chocolate milk, *Rah. aquatilis* can produce guaiacol within the expected shelf-life of the product, even without temperature abuse.

**Significance and Impact of the Study:** This is the first report that the coliform *Rah. aquatilis* can produce guaiacol in refrigerated chocolate milk products.

**INTRODUCTION**

Over the past 10 years, several incidents of smoky/phenolic odours in dairy products such as chocolate ice cream and vanilla yoghurt have been reported (Saxby 1993; Whitfield 1998). In all cases guaiacol (2-methoxyphenol) was identified as the microbial metabolite; however, the micro-organisms responsible for the formation of this compound were not identified. Recently, samples of chocolate milk with a smoky/phenolic odour were received in our laboratory for chemical and microbiological examination. Again, guaiacol was identified as the compound responsible for the odour but unlike earlier studies, the micro-organism responsible for the spoilage was also identified. This paper describes the isolation and identification of this bacterium, and reports studies to identify the precursor of guaiacol in chocolate milk.

**MATERIALS AND METHODS**

Several samples of pasteurized chocolate milk with a smoky/phenolic odour were received from a commercial processor in 200 ml cartons. The samples were from part of one day’s production and the odour had developed under normal refrigerated storage. A sample of chocolate milk not affected by the odour was also supplied. All samples were within the shelf-life (13 d) stated on the packs. The samples were stored overnight at 1°C and analyses commenced the following day. Only one spoiled and one unspoiled sample were tested microbiologically. Cartons containing UHT chocolate milk and UHT full cream (white) milk utilized in media and in the inoculation studies were purchased from a local retail outlet.

**Microbiological methods**

Microbiological growth media were purchased from Oxoid Ltd, Basingstoke, RG24–8PW, UK. Chocolate milk agar was prepared from double-strength nutrient agar (sterilized by autoclaving) and UHT chocolate milk which was steamed for 15 min. Both solutions were cooled to around 50°C, combined in equal volumes, and poured into Petri dishes.

Samples were mixed to ensure homogeneity and serial dilutions were prepared with 0.1% (w/v) peptone solution. Samples and dilutions were inoculated onto chocolate milk agar plates (single plates from each dilution), using the spread plate technique and 0.1 ml inocula. Sets of plates were
incubated at 8–9°C for 11 d and 19–20°C for 5 d. The pH values of the spoiled and control milks were measured.

Plates were examined for growth and for the presence of an odour similar to that of the spoiled milk. Representatives of all colony types from plates exhibiting the odour from both incubation temperatures and containing between 1 and 50 colonies, were inoculated onto individual plates of chocolate milk agar. Plates were incubated at 30°C for 5 d and examined for the odour. The isolates producing the odour were retained and those not producing the odour were discarded. The percentage of odour-producing isolates amongst the total microflora was calculated. The odour-producing isolates were Gram stained and their microscopic morphology examined. They were tested for their catalase and oxidase reactions, and their ability to grow at 37°C. They were tested using the API 20 E and 50 CHE systems (bioMérieux, Marcy l’Etoile, France) incubated at 37°C. Additional tests for motility, Voges-Proskauer (VP), Simmons citrate and methyl red were conducted according to methods prescribed by Krieg and Gerhardt (1994) for motility only, and Smibert and Krieg (1994) for the remaining tests. Motility tests were conducted at 25°C and 36°C, Simmons citrate tests at 30°C and methyl red tests at 30 and 37°C. The isolate was tested for growth on violet red bile agar (VRB) incubated overnight at both 30°C and 37°C.

Studies were conducted to induce odour formation in UHT milk. The odour-producing isolate was grown overnight in nutrient broth incubated at 30°C. A 1:100 dilution of the culture was prepared in peptone solution. From this suspension 0.1 ml aliquots were inoculated into duplicate 400 ml samples of UHT chocolate milk and UHT full cream (white) milk, with and without vanillin (Quest International, The Netherlands), added at 27 mg l⁻¹, the concentration present in the refrigerated chocolate milk. The target inoculum was 5 × 10⁴ colony forming units (cfu) ml⁻¹ milk for all samples. Uninoculated samples of both milks were also prepared. All samples were mixed and then incubated at either 4–5°C or 8–9°C for 6 d. They were re-mixed and 20 ml samples transferred aseptically to sealed containers for analysis by gas chromatography/mass spectrometry (GC/MS) the same day. From the remainder of each incubated sample serial dilutions were prepared and plated in duplicate onto brain heart infusion agar, using the spread plate technique and 0.1 ml inocula. Plates were incubated at 30°C for 2 d and the colonies counted.

**Collection of headspace volatiles from milk samples**

Measured volumes (10 ml) of either spoiled, control, or inoculated milk were placed in crimp-top 27 ml headspace vials (Alltech Associates Inc, Deerfield, IL, 60015–1899, USA) containing a magnetic stirrer bar. The internal standard, 2-ethoxyphenol (Aldrich Chemicals Inc., Milwau-kee, WI, 53233, USA), 1 µg in 100 µl of water, was added and the vials sealed. The volatile compounds were isolated by solid phase microextraction (SPME) using a fibre coated with 75 µm polydimethylsiloxane–carboxen (Supelco, Belle-fonte, PA, 16823–0048, USA) according to the method of Nilsson et al. (1996). The conditions used for the isolation of the milk volatiles had been previously shown to favour the recovery of a range of volatile compounds from milk (Marsili 1999).

**Analysis of headspace volatiles by GC/MS**

All analyses were performed on a Hewlett-Packard HP 5972 mass selective detector (MSD) interfaced to a HP 5890 series II GC controlled by a HP G 1034 C MS ChemStation (Hewlett Packard, Palo Alto, CA, 94304–1185, USA). The gas chromatograph (GC) was fitted with a fused silica capillary column (25 m long × 0.2 mm i.d.) coated with a 0.25 µm film of bonded siloxane HP-5 (Hewlett Packard). The volatile compounds from the milk samples were desorbed from the SPME fibre at 270°C into the introducer of the GC while the GC oven was held at 35°C. The headspace volatiles were analysed as previously described by Whitfield et al. (2000) with the exception that the MSD was operated in the selected ion monitoring mode (ions 120, 109 and 81 were monitored). Data generated by the MSD were acquired, stored and processed using the ChemStation software. When the MSD was operated in the full scan mode, the mass spectra of individual GC peaks were compared with reference spectra in the US National Institute of Science and Technology library of standard reference spectra.

Quantitative data were obtained by comparing the GC/MS chromatogram peak areas with the area of the internal standard, 2-ethoxyphenol. A calibration curve was produced by the analysis of three solutions of chocolate milk (10 ml) containing 0.1, 0.5 and 1 µg of guaiacol (Aldrich Chemicals) and 1 µg of internal standard. The calibrations were performed in triplicate. The concentrations of guaiacol are reported in ng ml⁻¹. The detection limit for this compound was estimated to be 0.05 ng ml⁻¹ of milk, based on 3 times background noise.

**Sensory assessment**

The odour threshold concentration of guaiacol in chocolate milk was determined as follows. A stock solution of guaiacol in methanol (100 µg 10 ml⁻¹) was prepared and aliquots of this solution (1, 2, 5, 10, 20 and 100 µl) were added to samples of control chocolate milk (20 ml) in 30 ml McCart-
ney bottles. After a thorough mixing, the solutions were assessed for smoky/phenolic odours by an informal panel of six persons. The data was interpreted as a plot of proportional positive response against log concentration and the threshold concentration was determined by linear calibration (Snedecor and Cochran 1989). The threshold was defined as the concentration at which there was a two-thirds positive response.

RESULTS

Microbiology

Counts from chocolate milk agar plates incubated at 8–9°C and 19–20°C and pH values of the initial spoiled and control milk samples are shown in Table 1.

A mixed microflora was detected in both milks. High numbers of bacteria, at levels normally associated with spoilage, were detected in the spoiled sample at both incubation temperatures. Lower bacterial numbers, more typical of unspoiled pasteurized milk, were detected in the control sample. At least four different types of bacteria were present in the spoiled milk, and at least three types in the control milk. A higher proportion of odour-producing colonies was detected in the spoiled milk on the plates incubated at 8–9°C compared with those incubated at 19–20°C. None were detected in the control milk. The limit of detection was 10 cfu ml⁻¹ milk.

The odour producing isolate was a Gram-negative, oxidase negative, catalase positive, rod shaped bacterium which was capable of growth at 37°C. It displayed colony morphology on VRB agar typical of the coliform group of organisms, when incubated at either 30°C or 37°C. The API 20 E identified the isolate to be either Pantoea spp. (42% probability) or Rahnella aquatilis (78-4% probability). The API 50 CHE test identified it as Rahn. aquatilis (95-2% probability) or Pantoea spp. (47% probability). Table 2 shows the results of selected tests from the API and additional tests. All except VP were typical of those shown by strains of Rahn. aquatilis. Holt et al. (1994) state that most Rahn. aquatilis strains are VP positive, however, up to 10% may be VP negative, the result shown by our isolate. The results therefore confirm the identity of the organism as Rahn. aquatilis.

Counts of bacteria in UHT milks incubated for 6 d at 4–5°C or 8–9°C are shown in Table 3. The inoculum was approximately 5 x 10⁵ cfu ml⁻¹ milk. The odour was detected in inoculated chocolate milks only, but all inoculated milks supported growth of the organisms. No growth was detected in the uninoculated controls. The limit of detection was 5 cfu ml⁻¹ milk.

Table 1 Bacterial counts, percentage of smoky/phenolic odour-producing organisms, pH and guaiacol concentrations in single samples of spoiled and unspoiled chocolate milk

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Guaiacol* (ng ml⁻¹)</th>
<th>Plates incubated at 8–9°C</th>
<th>Plates incubated at 19–20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Counts (cfu ml⁻¹)</td>
<td>% odour producers</td>
</tr>
<tr>
<td>Spoiled</td>
<td>5.0</td>
<td>700–900</td>
<td>5 x 10⁵</td>
<td>57</td>
</tr>
<tr>
<td>Control</td>
<td>6.5</td>
<td>ND†</td>
<td>4 x 10⁵</td>
<td>nd‡</td>
</tr>
</tbody>
</table>

*Odour threshold 43 ng guaiacol ml⁻¹ chocolate milk.
†ND, Not Detected at a detection limit of 0.05 ng ml⁻¹ milk.
‡nd, Not Detected at a detection limit of 10 cfu ml⁻¹ milk.

Table 2 Selected characteristics of Rahnella aquatilis* and Pantoea spp.* compared with those of the milk isolate

<table>
<thead>
<tr>
<th>Test</th>
<th>Rahnella aquatilis</th>
<th>Pantoea spp.</th>
<th>Milk isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility at 25°C</td>
<td>90–100% strains motile</td>
<td>90–100% strains motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Motility at 36°C</td>
<td>90–100% strains non motile</td>
<td>90–100% strains motile</td>
<td>Non motile</td>
</tr>
<tr>
<td>Methyl red</td>
<td>76–89% strains positive</td>
<td>26–75% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>90–100% strains positive</td>
<td>90–100% strains positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Simmons citrate</td>
<td>90–100% strains positive</td>
<td>90–100% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>76–89% strains positive</td>
<td>0–10% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Lactose</td>
<td>90–100% strains positive</td>
<td>0–25% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Raffinose</td>
<td>90–100% strains positive</td>
<td>0–10% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>90–100% strains positive</td>
<td>0–10% strains positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Yellow pigment</td>
<td>90–100% strains negative</td>
<td>26–100% positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Adapted from Holt et al. (1994).
Table 3 Inoculation studies in UHT chocolate milk and UHT white milk with and without vanillin stored for 6 d

<table>
<thead>
<tr>
<th>Milk type</th>
<th>Inoculated</th>
<th>Count (cfu ml⁻¹)*</th>
<th>Off-odour†</th>
<th>Guaiacol (ng ml⁻¹)*</th>
<th>Stored at 4–5°C</th>
<th>Stored at 8–9°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate</td>
<td>Yes</td>
<td>6 x 10⁴</td>
<td>Yes‡</td>
<td>50–70</td>
<td>9 x 10⁸</td>
<td>Yes</td>
</tr>
<tr>
<td>Chocolate</td>
<td>No</td>
<td>nd***</td>
<td>No</td>
<td>ND††</td>
<td>nd</td>
<td>No</td>
</tr>
<tr>
<td>White</td>
<td>Yes</td>
<td>5 x 10⁴</td>
<td>No</td>
<td>ND</td>
<td>6 x 10⁸</td>
<td>No</td>
</tr>
<tr>
<td>White</td>
<td>No</td>
<td>nd</td>
<td>No</td>
<td>ND</td>
<td>nd</td>
<td>No</td>
</tr>
<tr>
<td>White + vanillin</td>
<td>Yes</td>
<td>7 x 10⁴</td>
<td>No</td>
<td>ND</td>
<td>4 x 10⁸</td>
<td>No</td>
</tr>
<tr>
<td>White + vanillin</td>
<td>No</td>
<td>nd</td>
<td>No</td>
<td>ND</td>
<td>nd</td>
<td>No</td>
</tr>
</tbody>
</table>

*Mean values from duplicate samples.
†Odour threshold 43 ng guaiacol ml⁻¹ chocolate milk.
‡The odour was described as smoky/phenolic.
***nd, Not Detected at a detection limit of 5 cfu ml⁻¹ milk.
††ND, Not Detected at a detection limit of 0.05 ng guaiacol ml⁻¹ milk.

Chemistry and sensory analysis

Quantitative data for the concentration of guaiacol detected in samples of spoiled milk and inoculated UHT chocolate milk are given in Tables 1 and 3. In these samples, guaiacol was identified by its mass spectrum and GC retention time and by comparison with an authentic sample of the compound. The detection limit was 0.05 ng guaiacol ml⁻¹ milk.

A comparison of the GC/MS chromatograms of the volatile components of spoiled, inoculated and control samples of milk showed that guaiacol was only present in the samples of spoiled chocolate milk and in the inoculated samples of UHT chocolate milk. Guaiacol was not detected in either the control samples or the inoculated samples of white milk with or without vanillin.

The odour threshold concentration (OTC) was determined by linear calibration (Snedecor and Cochran 1989) to be 43 ng guaiacol ml⁻¹ chocolate milk.

DISCUSSION

A Gram-negative, psychrotrophic, coliform bacterium identified as *Rah. aquatilis* was shown to produce guaiacol in chocolate milk stored at acceptable refrigeration temperatures (4–5°C) and at abuse temperatures (8–9°C). Coliforms are heat-sensitive organisms which are inactivated by conventional pasteurization processes; their presence in pasteurized milk therefore indicates post-process contamination. Inoculation studies showed that relatively low initial numbers of the bacteria (5 x 10² cfu ml⁻¹) could produce a detectable smoky/phenolic odour in 6 d at acceptable and abuse refrigeration temperatures. Accordingly, if this bacterium is present in chocolate milk, production of guaiacol could be expected in milks stored without temperature abuse within the expected shelf-life of the product (13d).

The natural habitat of *Rah. aquatilis* is water but the organism has also been isolated from clinical samples (Farmer 1984; Funke and Rosner 1995) and foods (Gras et al. 1994; Lindberg et al. 1998), including pasteurized milk (Lindberg et al. 1998; Whitfield et al. 2000), pasteurized cream (Lindberg et al. 1998) and cottage cheese (Davey and Eyles 1992). It is likely that this bacterium is a frequent contaminant of foods, but investigators fail to differentiate it from other coliforms.

Currently, *Rah. aquatilis* is considered to be of little public health significance although a gene encoding for a heat labile toxin has been identified in a single strain isolated from fish (Lindberg et al. 1998). Nevertheless, the current study has shown that the presence of this bacterium in some chocolate dairy products can cause spoilage through the development of an unacceptable odour.

The smoky/phenolic odour of guaiacol is well established in coffee (Mayer et al. 1998) and barley malt (Fickert and Schieberle 1998) but it is probably better known for its production of off-odours in fruit juices (Pettipher et al. 1997; Jensen 2000), chocolate ice cream (Saxby 1993) and vanilla yogurt (Whitfield 1998). In roasted products, guaiacol is formed by thermal decomposition of phenolic precursors, whereas in fruit juice and dairy foods it is a product of microbial metabolism. Micro-organisms known to produce guaiacol include Bacillus megaterium (Crawford and Perkins Olson 1978), Pseudomonas acidovorans (Vicuña et al. 1987), Alcyclobacillus acidoterrestris (Pettipher et al. 1997), an actinomycete Streptomyces setonii (Pometto et al. 1981) and the yeast Rhodotorula rubra (Huang et al. 1993).

Few pathways have been established for the metabolic production of guaiacol. Studies have shown that vanillic acid...
can be metabolized to guaiacol by several strains of \textit{B. megaterium} (Crawford and Perkins Olson 1978) and by a strain of \textit{S. setonii} (Pometto et al. 1981). Vanillin has also been metabolized to guaiacol by an unidentified species of \textit{Streptomyces} (Lefebvre et al. 1983) and ferulic acid by \textit{Rho. rubra} (Huang et al. 1993). In both of these metabolic pathways, vanillic acid was the immediate precursor of guaiacol. Vanillin is a major component (27 mg l$^{-1}$) of the flavouring of chocolate milk. However, our attempt to show the metabolism of vanillin to guaiacol in white milk by \textit{Rah. aquatilis} was unsuccessful (Table 3). Accordingly, this bacterium is unable to perform this biotransformation in white milk, even in the presence of vanillin. This result, coupled with findings of other workers (Crawford and Perkins Olson 1978; Pometto et al. 1981) would suggest that vanillic acid (an oxidation product of vanillin) is the likely precursor of guaiacol in chocolate milk. Studies are currently in progress to establish the precursor of guaiacol in this product.

**ACKNOWLEDGEMENTS**

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