Characterization of a heat resistant mesophilic bacillus species affecting quality of UHT-milk - a preliminary report - [1)]

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1. Introduction

Heat resistant mesophilic aerobic sporeformers (HRS) were detected in UHT-milk from Italy and Austria in 1985. They first occurred in Germany in 1990 after the new Milk Ordinance (EC-Directive 85/397 (1), Milchverordnung 23.6.1989 (2)) came into effect. According to these regulations the maximum colony-count in UHT-milk after incubation of the packages for 15 days at 30°C must be less than 10 cfu/0.1 ml. Dairies and food inspection services were most surprised to find colony-counts up to 10^5 in a product which was believed to be practically sterile ("commercial sterility"). Nowadays the problem is known in a number of dairies in Germany and other European and extraeuropean countries, and, in part has led to severe consequences for certain dairies (Table 1).

Tab. 1: Chronology and incidence of heat resistant spore formers (HRS) in UHT-milk production

1985	First detection in Italy and Austria
1990	First detection in Germany
1992	2 dairies in Italy, one dairy in Germany nearly closed by local authorities
1995	1 UHT-production in a dairy closed in Italy by local authority, 1 dairy going to be closed in Italy

Confirmed cases until 1995:

Country	no. of dairies
Germany	16
Italy	17
France	16
Benelux	2
Spain	1
Extraeuropean	2

2. Biological characterization

The HRS are Gram-positive to Gram-labile rods of 3.4-5.2 µm length and 0.7 µm width. In older cultures, as well as in cultures after several agar-passages, filaments of up to 40 µm can be observed. According to experts this is a sign that the organisms are adapted to well to the medium. The first isolates from UHT-milk, however, always appear as regular rods, as mentioned above. Endospores are located terminally, size 1.7 x 0.7 µm, with the sporangium not swollen. The sporulation rate usually is very low, approximately 1:1000.

[1)] according to a paper presented at the „IDF-Symposium on Heat Treatments and Alternative Methods", Vienna 6-8 Sept. 1995

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All strains are motile by flagellae. Colonies on nutrient agar are greyish-white, occasionally with an orange centre, 1-2 mm in diameter, round, with a slightly rough surface and undulated margin. On blood agar no haemolysis occurs. Growth was observed at temperatures between 20 and 52°C with an optimum between 35 and 42°C. Oxygen is strictly required. To reach a colony count of 10^5/ml in UHT-milk a minimum incubation time of 2-5 days at 30°C is required. Hydrocarbons are not required, they are used very slowly or even not at all. Enzymes such as cytochromeoxidase, catalase and proteases are present.

3. Taxonomy

The taxonomy of the organisms is not yet clear. Certainly they are members of the genus Bacillus due to strictly aerobic growth and the formation of endospores. Several working groups were involved in identifying the HRS using genetical and conventional microbiological methods (Table 2).

Tab. 2: Taxonomy of HRS, working groups involved

Working group	method	result
Royal Institute for Technology, Stockholm (M. Úhlen)	16 S rDNA	ongoing*
German Collection of Microorganisms, Braunschweig (E. Stackebrandt)	16 S rRNA	ongoing*
Netherlands Institute for Dairy Research, Ede (N. Klijn)	16 S rRNA	87% homology to *B.badius*
Harriot Watt University, Edingburgh (F. Priest)	genotypic and phenotypic taxonomy	ongoing*
Tetra Pak Research, Stuttgart (F. Lembke)	FTIR-Spectroscopy	ongoing*
Federal Dairy Research Centre, Kiel (P. Hammer)	phenotypic taxonomy	ongoing* (closest relation to *B.badius*)

* new taxons expected

As an example of a genetical classification, a dendrogram achieved by 16S rDNA hybridization was elaborated by the group of M.ÚHLEN (3) (Fig. 1).

This dendrogram and the results of the other groups using these methods showed that the HRS group is not absolutely homogeneous. The establishment of at least two new taxa which, however, will be closely related is expected by the experts. Biochemical reaction patterns of the HRS as derived in our working group indicate too that there are two different groups of HRS (Table 3). Growth patterns are quite similar in both groups; however, carbohydrate utilization in the smaller group of these strains is significantly different.

An important result of the taxonomical work is that the organisms certainly are not members of a hitherto known group of pathogenic bacilli.

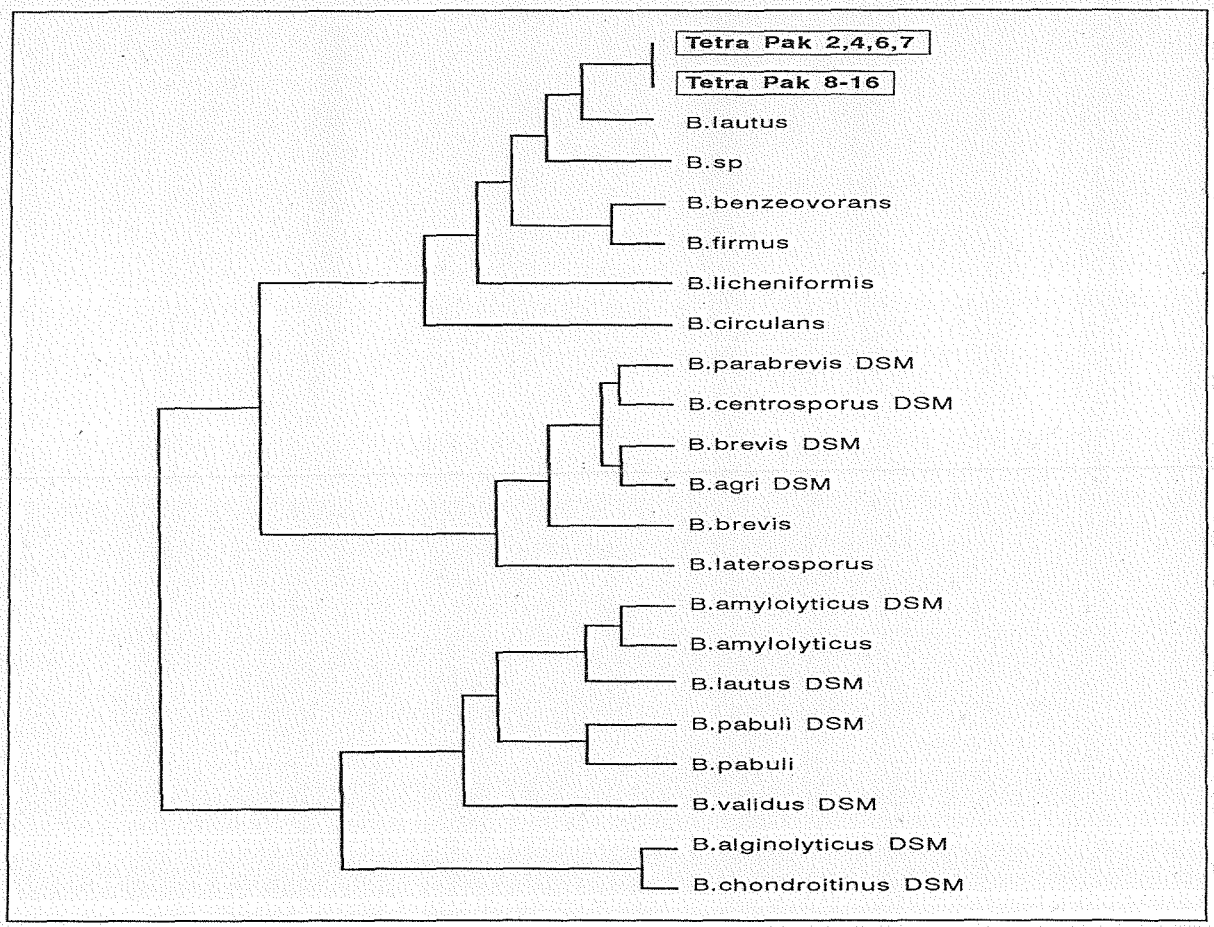

Fig. 1: Genotypic taxonomy of HRS, 16 S rDNA dendrogram (M. Úhlen, B. Petterson - KTH Stockholm)

Tab. 3: Biochemical reaction pattern of the HRS

Strain	45 °C	50 °C	55 °C	NaCl 5 %	NaCl 7 %	NaCl 10 %	pH 6.8	pH 5.7	glucose	arabinose	lactose	sucrose	trehalose	raffinose	inulin	mannitol	sorbitol	aesculin	arginine	xylose	starch	gelatin
J 16	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
18	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N III 10/1	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
N IV 16/10	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
N V 16/6	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
N V 9/8	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
N VI 14/4	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
TP 1147	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP 1248	+	+	-	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-
TP 1252	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
H_1 41	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
32	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	+
TP 1168	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-
H_2 34	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	-	-	+

4. Products involved

Products involved, according to present knowledge, are UHT-milk, UHT-cream, chocolate milk and milk powder. The products are not affected in their sensoric properties. Chemical/physical changes are not detectable except apart from, very rarely, a heat induced coagulation after prolonged storage in bottles with a low oxygen barrier. In such plastic bottles the development of a pink colour has been described. Colony counts of the HRS never reached more than 10^5 cfu/ml. Even prolonged incubation up to 78 days at 30°C did not enhance the count.

HRS contamination occurs in nearly all types of UHT-equipment of all suppliers. However, in direct heating systems the problem is much easier to solve than in indirect systems, because in direct systems heating conditions can be enhanced without significant increase of lactulose content.

UHT-products other than milk may also be affected but no problems have been reported recently.

5. Detection

The isolation of the HRS from contaminated milk is possible after several days of pre-incubation of the milk at 30-40°C on a nutrient agar containing vitamin B_{12} (1 mg/l). Due to suppressed growth, HRS can not be isolated from UHT-milk packages containing any other contaminating flora. Raw milk must be autoclaved for 5 min in a flat bottom flask with a milk layer less than 1 cm to preselect HRS spores, followed by incubation at 35-37 °C for 3-5 days for enrichment. For faster detection a gene probe technique as well as a PCR method are under development by several of the working groups mentioned above.

6. Contamination sources

Until now the natural reservoir of the HRS is not known. Probably UHT-milk has been occupied as an ecological niche. The source of contamination is not clear either. It is obviously not a problem of recontamination in the UHT-processing/ packaging line of the dairy as the HRS could be isolated from a bypass directly after the heating section of an indirect heating device. Contamination of raw milk in the preprocessing area is not very likely either because often only single storage tanks are contaminated possibly due to a certain tanker tour. Surprisingly the HRS-problem in Germany is reduced to the south, and within one dairy only to certain areas of milk collection. This year HRS were detected for the first time in ex-farm milk. However, the means of contamination at the farm is also not clear. Only in one or two of 120 feed samples of corn silage, grass silage and sugar beet were high numbers of HRS spores detected. Thus contamination of raw milk at the farm level via feed or milking equipment is most likely but not yet proven.

Another means of contamination is discussed with respect to reprocessing of contaminated lots of UHT-milk. Because the organisms truly can survive a normal indirect UHT-treatment, a reprocessing of about 1000 packages with a higher spore content will be sufficient to contaminate a whole days' production at a level of one spore/l, which is regarded as the common contamination level observed.

A third means is possible by processing of contaminated milk powder. HRS were recently found in UHT-milk and in the milk powder used for processing in an extraeuropean country.

7. Heat resistance

To date, research concerning the possible extreme heat resistance of the HRS has not yielded conclusive results. The results of the laboratories involved are quite different (Table 4).

Tab. 4: Heat resistance of HRS

Institute	Result
Canning Research Institute, Campden (UK)	D_{100} = 5.09 min
Institute for Food Technology, Weihenstephan (D)	D_{121} = 8.3 - 34 sec
Tetra Pak, Lund (S)	Fo < 10 min (contaminated milk) Fo > 68 min (pilot plant, production plant)
Tetra Pak Research, Stuttgart (D)	D_{98} > 60 min; $D_{120} \approx$ 10 min
Netherlands Institute for Dairy Research, Ede (NL)	D_{126} = 1 min; D_{147} = 5 sec

Examinations of Tetra Pak in a production plant with a heavy HRS contamination showed that temperature/time combinations of 148°C/10 sec or 150°C/6 sec resulted in a satisfactory sterilizing effect. These heating conditions correspond to F_0 values of 69 and 66 minutes, respectively, however, calculated on the holding cell only.

The results of NIZO (4) indicate that the spores are extremely heat resistant in the UHT region but less exceptional than other bacilli at 126°C.

Concerning the different results of the laboratories involved it is quite likely that the heat resistance of laboratory cultured strains is lower than that of field strains in naturally contaminated milk, as most examinations under laboratory conditions did not show such extreme heat resistance of the HRS as in the "field trial" in one dairy plant. The morphological visible degeneration after several culture passages also indicates this.

Another problem of concern for the examination of the heat resistance is the low sporulation rate of the HRS which makes it difficult for investigators to reach satisfying spore counts.

8. Pathogenicity

When the HRS-problem came to light the most important question to all the individuals involved and/or responsible was whether or not the organisms might be harmful to the consumer (risk assessment of the hazard). Again several laboratories were involved in testing the HRS for pathogenicity or cytotoxicity.

Pathogenicity tests in mice at the Inveresk Research Institute in Scotland (5) using live cultures and sterile supernatants in oral and intravenous dosing routes did not show any pathogenic or toxic effects in the animals. Cytotoxicity tests on four different cell lines at the Institute for Virus Research in Eutin-Sielbeck, Germany (6), did not show toxic effects either. However, in both studies only one or two HRS-strains were tested.

In our laboratory we tested at least 15 HRS-strains on VERO B4-cells by morphological evaluation and release of lactate dehydrogenase (LDH) (Table 5). Only a combination of LDH-release >10% and a morphologically evaluated damage of cells was defined as a "positive" reaction. Using this standard almost no cytotoxic effects could be observed.

Tab. 5: **Pathogenicity of HRS - Cytotoxicity on VERO B4-cells, morphological evaluation and release of lactate dehydrogenase (LDH)**

sterile supernatants of strain		morphological evaluation				LDH-release		
		n effect	no damaged	slightly	damaged	n	< 10%	≈10%
J	16	9	5	3	1	6	5	1
	18	9	7	1	1	6	6	0
	42	7	7	0	0	6	6	0
N III	10/1	9	7	1	1	6	6	0
N IV	16/10	8	4	3	1	6	5	1
N V	16/6	8	6	0	2	6	6	0
N V	9/8	10	6	3	1	6	5	1
N VI	14/4	9	8	1	0	6	5	1
TP	1147	8	5	0	3	6	5	1
TP	1248	9	8	0	1	6	6	0
TP	1252	7	6	1	0	6	6	0
	32	8	7	1	0	6	6	0
TP	1168	7	6	1	0	6	6	0
H_2	34	9	6	1	2	6	6	0
B.cereus		9	1	1	7	6	2	4

However, on cell cultures only toxic effects can be examined. To use a model of higher impact embryonized chicken eggs were inoculated to test the HRS for pathogenicity. Those investigations are still ongoing. Preliminary results, however, indicate that no pathogenic effects can be confirmed.

9. Conclusions

In summary it can be concluded that according to our present knowledge, the HRS do not form a pathogenic bacillus group, so they are less important from the hygienic point of view but most important from a technological perspective. Contaminated milk is suitable for consumption, but does not fulfil legal requirements and the typical properties of that which is called UHT-milk.

Research concerning taxonomy, detection methods and contamination sources have to be continued. The technological problems due to the extreme heat resistance can be resolved and are, in part, by using recently developed heating devices with a combination of indirect and direct heating (7).

Acknowledegement

Most of the research work presented here was initiated and supported by Tetra Pak Research in Stuttgart (Dr. Fritz Lembke).

10. References

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11. Zusammenfassung

Hammer, P., Lembke, F., Suhren, G., Heeschen, W.: **Charakterisierung einer die H-Milch-Qualität beeinflussenden hitzeresistenten, mesophilen Bacillusspecies - Vorläufige Mitteilung**. Kieler Milchwirtschaftliche Forschungsberichte **47** (4) 303-311 (1995)

06 Hitzeresistente Bacillusspecies (H-Milchqualität)

Hitzeresistente Sporenbildner (HRS) wurden in Deutschland erstmalig nach Inkrafttreten der Milchverordnung vom 23.6.1989 (EG-Richtlinie 85/397) aus UHT-Milch isoliert. Nach diesen Verordnungen darf die Keimzahl in einer 15 Tage bei 30°C bebrüteten Packung 10 KbE/0,1 ml nicht überschreiten ("kommerzielle Sterilität"). Inzwischen ist das HRS-Problem in einer Vielzahl von Molkereien im In- und Ausland bekannt.

Die biologischen Eigenschaften der HRS in bezug auf Morphologie, Vermehrungsbedingungen und biochemisches Reaktionsmuster werden vorgestellt. Eine taxonomische Einordnung ist derzeit noch nicht möglich. Durch Untersuchungen verschiedener Arbeitsgruppen mit geno- und phänotypischen Methoden werden sich vermutlich noch in diesem Jahr zwei neue Spezies etablieren lassen.

Ein Vorkommen von HRS ist in UHT-Milch und -sahne, Kakao und Milchpulver beschrieben. Kontaminationen sind bisher in allen Arten von Erhitzeranlagen aller Hersteller aufgetreten. Als Nachweisverfahren ist derzeit nur eine mikrobiologische Methode mit einem Autoklavierschritt zur Vorselektion der Keime beschrieben. Schnellere Verfahren mittels Gensonde bzw. PCR befinden sich in der Entwicklung. Durch das relativ unempfindliche und zeitraubende Nachweisverfahren sind bisher die Kontaminationsquellen weder auf Molkerei- noch auf Hofebene aufgeklärt worden. Möglicherweise sind auch Wiederverwertung kontaminierter UHT-Milch und Herstellung von UHT-Milch aus kontaminiertem Milchpulver als Ursache nicht auszuschließen.

Die vermutlich extreme Hitzeresistenz ist mit unterschiedlichem Ergebnis in zahlreichen Labors untersucht worden. Eine in Laboranlagen ermittelte, nicht als hoch anzusehende Resistenz ist vermutlich darauf zurückzuführen, daß Feldstämme in "natürlich" kontaminierter Milch offensichtlich wesentlich resistenter sind als mehrfach kultivierte Laborstämme.

Der wesentliche Gesichtspunkt im Hinblick auf den Verbraucherschutz ist die Frage nach möglichen pathogenen Eigenschaften. Sofort nach Bekanntwerden der Problematik eingeleitete Pathogenitätsversuche an Mäusen und im embryonierten Hühnerei sowie Zytotoxizitätsversuche auf verschiedenen Zellinien geben hierfür jedoch keine Anhaltspunkte.

Summary

Hammer, P., Lembke, F., Suhren, G., Heeschen, W.: **Characterization of a heat resistant mesophilic bacillus species affecting quality of UHT-milk - a preliminary report.** Kieler Milchwirtschaftliche Forschungsberichte **47** (4) 303-311 (1995)

06 Heat resistant bacillus species (UHT-milk quality)

Heat resistant mesophilic sporeformers (HRS) first occurred in Germany after the new Milk Ordinance (EC-Directive 85/397, Milchverordnung 23.6.1989) came into effect. According to these regulations a maximum colony count of 10 cfu/0.1 ml is required after 15 days incubation of the packages at 30°C. HRS reach colony counts up to 10^5/ml without changing milk composition or sensoric properties. The problem nowadays is evident in a number of dairies in several countries.

A biological characterization of the HRS including morphological patterns as well as growth conditions and biochemical properties is presented. The taxonomy is not yet clear. Several working groups are involved using genetical or phenotypical methods to establish a new taxon group.

Products involved are UHT-milk, UHT-cream, chocolate milk and milk powder. Contamination occurs in all types of UHT-equipment of several suppliers.

For detection of the HRS only a conventional autoclaving method is available, faster methods as gene probes or PCR are under development. Regarding the time consuming and insensitive detection method the way of contamination as well as the contamination sources are not clear. Possibilities at the dairy and the farm level are discussed, however, problems regarding reprocessing of contaminated UHT-milk or processing of contaminated milk powder are under consideration too.

The probably extreme heat resistance of the HRS was investigated by several laboratories. Results are differing, most probably because HRS-strains in naturally contaminated milk seem to be much more resistant than laboratory cultured strains.

With regard to the consumers safety investigations concerning the pathogenicity of the HRS started as early as the problem was known. Pathogenicity tests in mice and embryonized chicken eggs and cytotoxicity tests on different cell lines are presented. At this moment there is no evidence for any pathogenic or toxic properties of the HRS.

Résumé

Hammer, P., Lembke, F., Suhren, G., Heeschen, W.: **Caractérisation d'une espèce de bacille mésophile thermorésistant qui influe sur la qualité du lait UHT - rapport préliminaire.** Kieler Milchwirtschaftliche Forschungsberichte **47** (4) 303-311 (1995)

06 Espèce de baoille thermorésistant (qualité du lait UHT)

Des sporulés thermorésistants (ST) ont été isolés du lait UHT en Allemagne depuis l'entrée en vigueur de la nouvelle ordonnance laitière du 23 juin 1989 (directive 85/397 de la CEE). Selon ce règlement, le nombre de colonies ne doit pas dépasser 10 ufc/0,1 ml après incubation des produits dans leur emballage à 30°C pendant 15 jours («stérilité commerciale»). Depuis, le problème des ST est bien connu pour un grand nombre d'usines de plusieurs pays.

Les propriétés biologiques des ST, morphologie, conditions de multiplication, spectre de réactions biochimiques sont présentées. Actuellement, la classification taxinomique n'est pas encore très claire. Les recherches menées par plusieurs groupes de travail permettront vraisemblablement de définir encore cette année deux nouvelles espèces à l'aide des méthodes génotypiques et phénotypiques.

Les ST ont été détectées dans le lait UHT, la crème UHT, le lait chocolaté et la poudre de lait. Une contamination a été observée pour tous les types d'installations UHT de tous les fabricants. Pour détecter les ST, on dispose seulement actuellement de la méthode conventionnelle par autoclavage. Des essais ont donc été entrepris pour développer des méthodes plus rapides telles que les sondes génétiques ou la PCR. Avec la méthode traditionnelle, longue et non sensible, il n'était pas possible de définir clairement le mode et la source de contamination. Les possibilités de contamination au niveau de l'usine ou de l'exploitation sont discutées, ainsi que celles pouvant intervenir au niveau de l'utilisation de lait UHT contaminé ou de mise en œuvre de poudre de lait contaminée.

La haute résistance probable des ST a été étudiée par plusieurs laboratoires. Les résultats divergent, probablement du fait que les souches ST qui contaminent naturellement le lait sont beaucoup plus résistantes que les souches de laboratoire.

Les recherches concernant la pathogénicité potentielle des ST ont été conduites dès que le problème a été connu, dans un souci de protection du consommateur. Les résultats des tests de pathogénicité sur souris et œufs fécondés et des tests de toxicité sur différentes lignées cellulaires sont présentés. A l'heure actuelle, aucune pathogénéicité ou propriétés toxiques n'a été mise en évidence.