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Lactic acid bacteria associated with pig skin at pre- and post-scalding slaughter stages

Milchsäurebakterien auf der Haut von Schweinen vor und nach dem Brühprozess

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Summary

Lactic acid bacteria (LAB) play a role in meat fermentations but they also are specific spoilage bacteria in modified atmosphere packaged (MAP), cold-stored meats. Their origin and contamination in the meat processing environment is not yet fully understood. In this study, cutaneous LAB microflora of pigs at pre- and post-scalding slaughter stages was assessed. Sampling of 35 pigs was done at a large-scale abattoir. To avoid heavy faecal contamination, LAB were enriched from the skin of earlobes. One earlobe of a carcass was cut immediately after stunning and the other after scalding. Based on a preliminary study, three different enrichment strategies selecting either 1) LAB generally, 2) psychrotrophic LAB or 3) vancomycin-resistant *Leuconostoc-Weissella* group were used for LAB isolation. Enrichment strategies resulted in total of 157 LAB isolates which were identified using a numerical taxonomy library utilizing HindIII ribopatterns as operational taxonomy units. Fifty-eight and 32 % of the LAB obtained during pre- and post-scalding samples, respectively, were *Lactobacillus* species *Lactobacillus curvatus* being detected in 22 pigs. In addition, *Weissella* and *Lactococcus* were commonly recovered whereas only 5 *Leuconostoc* isolates were detected. Based on this result, porcine skin is not unequivocally a source of all LAB species which have been reported to grow in MAP pork meat products.

Keywords: Lactic acid bacteria, pork, slaughtering, pig skin, psychrotroph

Zusammenfassung

Milchsäurebakterien (LAB) spielen eine Rolle bei der Fermentation von Fleisch, aber auch als Fäulniserreger bei unter Schutzatmosphäre verpacktem, kühl gelagertem Fleisch (MAP). Ihr Ursprung bei der Fleischverarbeitung ist noch nicht vollständig geklärt. In dieser Studie wurde die kutane LAB Mikroflora von Schweinen vor und nach dem Brühprozess untersucht. Die Probenentnahme bei 35 Schweinen wurde in einem großen Schlachtbetrieb durchgeführt. Zur Vermeidung von starker fäkaler Kontamination wurden Milchsäurebakterien von der Oberfläche der Ohrhaut untersucht. Ein Ohr eines Tierkörpers wurde sofort nach der Betäubung abgeschnitten und das andere nach dem Brühen. Basierend auf einer Vorstudie wurden drei verschiedene Anreicherungs-Varianten für die Isolierung verwendet. Entweder 1. für gesamt LAB, 2. für psychrotrophe LAB oder 3. für die Vancomycin-resistente *Leuconostoc-Weissella*-Gruppe. Insgesamt wurden 157 Isolate gewonnen. Bei 58 % der vor dem Brühprozess entnommenen Proben und bei 32 % der nach dem Brühprozess entnommenen Proben wurden Laktobazillen nachgewiesen. *Lactobacillus curvatus* wurde in 22 Schweinen nachgewiesen. Darüber hinaus wurden *Weissella* und *Lactococcus* häufig gewonnen, während nur fünf *Leuconostoc*-Isolate nachgewiesen wurden. Basierend auf diesen Ergebnissen ist Schweinehaut nicht eindeutig die Quelle aller LAB Arten, die in MAP Schweinefleisch-Produkten nachgewiesen werden.

Schlüsselwörter: Milchsäurebakterien, Fleisch, Schlachtung, Schweinehaut, psychothroph

Introduction

Lactic acid bacteria (LAB) play a double role in meat products. They are common in meat fermentations but may also cause spoilage of modified atmosphere packaged (MAP), cold-stored meats. *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus sakei, Lactobacillus curvatus, Pediococcus pentosaceus* and *Pediococcus acidilactici* are the species most often related to manufacture of fermented meats (Jessen, 1995). Psychrotrophic LAB species associated with meat spoilage belong mainly to the genera *Lactobacillus, Leuconostoc, Weissella* and *Carnobacterium* (Borch et al., 1996; Korkeala and Björkroth, 1997; Samelis, 2006; Schillinger et al., 2006).

Contamination of cooked meats with LAB during processing has been studied (Björkroth and Korkeala, 1996; Björkroth and Korkeala, 1997b; Björkroth et al., 1998) but the role of meat animals as sources of LAB has thus far been limited to a study dealing with LAB associated with skin of broiler chicken (Vihavainen et al., 2007). The temperature of the pig intestine is too high to support the colonization of the intestinal mucosa by psychrotrophic LAB which usually do not grow at temperatures exceeding 37 °C (Korkeala et al., 1988; Björkroth et al., 2000; Koort et al., 2004a, 2004b). However, it is not known if pig skin harbours these LAB and acts as a source of psychrotrophic LAB contamination. It is also not know if beneficial fermenting LAB are present on pig skin playing later a role in artisanal meat fermentations.

The aim of the study was to characterize LAB associated with pig skin and to assess if the LAB species detected in skin of pork differ from the broiler chicken isolates. Because the preliminary tests had indicated low LAB-levels associated with pork skin amd broiler chicken (Vihavainen et al., 2007), three different enrichment strategies selecting either 1) LAB generally, 2) psychrotrophic LAB or 3) the *Leuconostoc-Weissella* group were applied in this study.

Materials and methods

Carcass samples

Both earlobes of 35 randomly-chosen pigs slaughtered at one large-scale Finnish pig abattoir (capacity of 60 to 80 million carcass kg annually with US export licence) were collected during one morning. The left earlobe was cut off during the bleeding of stunned and stuck animals. Subsequently, these carcasses were followed during the slaughter process and their right earlobes were cut off after scalding in hot water tanks and dehairing by singeing, drying and polishing taking place before carcass evisceration and meat inspection procedures. The earlobes were placed in sterile plastic bags in which they were immediately taken to a laboratory facility within the abattoir premises. Skin strips of equal sizes, approximately 4 cm^2 each, were cut with sterile instruments from the outside surface of the lobes and enriched for LAB.

Selective enrichment procedures of skin samples

All skin samples were studied as described by Vihavainen et al. (2007) to enrich LAB, psychrotrophic LAB and leuconostocs. Briefly, enrichment for LAB and psychrotrophic LAB was done in MRS broth incubated at 25 ºC overnight to 5 days and at 6 °C for 38 days, respectively. Leuconostocs were enriched by placing a strip into MRS broth containing 10 µg/ml vancomycin (Sigma, St. Louis, USA). One-hundred and 57 Gram-positive, catalase-negative isolates were obtained from pre- and post-scalding samples, respectively, and they were all subjected to species identification.

DNA isolation, ribotyping and identification of isolates from product and skin samples

Cell mass for DNA extraction was produced from pure colonies in MRS broth. DNA was isolated using a modified method of Pitcher et al. (Pitcher et al., 1989; Björkroth and Korkeala, 1996) with cell lysis solution containing mutanolysin (250 U/ml, Sigma, St. Louis, MO, USA) and RNase in addition to lysozyme (25 mg/ml, Sigma). Restriction endonuclease treatment of 8 µg of DNA was done using HindIII restriction enzyme (New England Biolabs, Beverly, MA, USA) as recommended by the manufacturer. HindIII provides species-specific patterns for various LAB within the genera of *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Weissella* (Björkroth and Korkeala, 1996, 1997a; Björkroth et al., 1998, 2000; Susiluoto et al., 2003; Koort et al., 2005; Schillinger et al., 2006; Vihavainen and Björkroth, 2007). DNA fragments were run in an agarose gel electrophoresis and the resulting fingerprint patterns transferred onto nylon membranes via Southern blotting in a vacuum blotting device (Vacugene, Pharmacia, Uppsala, Sweden). Ribotyping was performed using a mixture of five cDNA oligo probes (transcribed from 16 and 23S rRNA) double-labeled with digoxigen.

The membranes were hybridized at 53 °C overnight and detection of the digoxigenin label was performed as recommended by Roche Molecular Biochemicals. After scanning the membranes (HP ScanJet 4c/T tabletop scanner, Boise, ID, USA), the HindIII ribopatterns were numerically analyzed with Bionumerics 4.61 software (Applied Maths, St Martens-Latem, Belgium). The similarity between all pairs was expressed by the Dice coefficient correlation. Clustering by the unweighted pair-group method with arithmetic averages (UPGMA) was used in the construction of a dendrogram. Based on the use of internal controls in the database, pattern optimization of 0.6 % and band position tolerance of 1.5 were allowed. The ribotype patterns were compared with corresponding patterns of over 500 LAB type and reference strains in the LAB database of the Department of Food Hygiene and Environmental Health. The isolates were identified based on the locations of type strains in the clusters. Reliability of the species-specific clusters has been evaluated in several polyphasic taxonomy studies of LAB (Björkroth et al., 2000, 2002; Koort et al, 2004a, 2004b, 2005, 2006).

Results

LAB growth in samples

All 70 earlobes resulted in growth but no growth was obtained in after-scalding enrichment for psychrotrophic LAB (MRS incubated at 6 °C). Table 1 shows which LAB species were detected and how the isolates were divided within these species in each three enrichment analyses. Altogether 20 species-specific clusters were obtained (Fig. 1) when the HindIII ribopatterns of all 157 isolates were subjected to the numerical analysis using the database. Twelve and 27 % of the isolates originating from the pre- and post-scalding skin samples, respectively, remained unknown.

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LAB in pre-scalding samples

L. curvatus was the species detected most commonly among the isolates in the pre-scalding samples. Among the total of 35 pigs, this species was detected in 22 individuals. Most isolates recovered from MRS-broth with vancomycin were lactobacilli, mostly *L. curvatus,* while the rest belonged to the genera of *Weissella* and *Leuconostoc.* These LAB species have either been reported to be intrinsically glycopeptide resistant or resistant to high vancomycin levels (Björkroth and Holzapfel, 2003; Danielsen and Wind, 2003). Of the isolates recovered from the plain MRS broth incubated at 6 °C, the majority were identified as lactobacilli (mostly *L. curvatus* and *Lactobacillus fuchuensis*), and none as leuconostocs. Isolates recovered from the plain MRS-broth incubated at 25 °C were either lactobacilli (*L. curvatus, Lactobacillus sakei* or *Lactobacillus plantarum* or *Lactobacillus brevis*), weissellas, lactococci *(Lactococcus garviae),* pediococci or leuconostocs *(Leuconostoc lactis).*

LAB in post-scalding samples

Scalding, singeing and polishing of the carcasses altered the microbiota. In samples taken after the scalding process, the prevailing species in plain MRS incubated at 25 °C was *L. plantarum* and *Weissella thailandensis.* Lactococci *(L. garviae),* pediococci and streptococci were also detected. The selective vancomycin enrichment procedure yielded no leuconostocs in the post-scalding samples, only lactobacilli, weissellas and pediococci were recovered. Psychrotrophic species *L. sakei, L. curvatus* and *L. fuchuensis* were not detected by any of the enrichment method. However, one psychrotrophic species, *Lactobacillus oligofermentans,* was detected both in the psychrotrophic enrichment of a pre-scalding sample and after scalding in two vancomycin-enriched samples. All 3 *L. oligofermentans* isolates were from different pigs.

Discussion

Psychrotrophic LAB have been reported to cause spoilage of pork meat products. Schillinger and Lücke (1986) detected *L. sakei/curvatus, Carnobacterium divergens* and *Carnobacterium maltaromaticum (Lactobacillus carnis)* in vacuum-packaged pork stored at 2 °C. While characterizing LAB in marinated vacuum-packaged pork, Schirmer et al. (2009) detected mainly *Lactobacillus algidus, L. sakei, L. curvatus, C. divergens, C. maltaromaticum, Leuconostoc mesenteroides* and *Leuconostoc carnosum* and reported *L. algidus* and *Le. mesenteroides* to produce the most unpleasant sensory changes in inoculation studies. When the species detected in the skin samples of the current study are compared with the previous studies dealing with the spoilage LAB, certain species can be associated both with the end products and the skin of the 35 pigs studied. Meat-associated lactobacilli *L. curvatus* and *L. sakei,* were present in the pre-scalding skin samples but they were not detected post-scalding. *L. fuchuensis,* which is a species closely rela-

TABLE 1: *Number of a total of 157 isolates originating from slaughter line samples of pig skin and distribution between lactic acid bacterium species.*

		Number of isolates (%)						
		Before scalding				After scalding		
	RT[a]	V[b]	P[c]	MRS[d]	Total	V[b]	MRS[d]	Total
Lactobacillus		**19**	**26**	**13**	**58 (58)**	**11**	**7**	**18 (30)**
L. curvatus	A	9	15	9	33			
L. sakei	Ba-d	2	3	2	7			
L. plantarum	Ca-c	4		1	5	5	6	11
L. brevis	Da-b	3		1	4	2		2
L. fuchuensis	Ea-b		7		7			
L. oligofermentans	Fa-e		1		1	2		2
L. farciminis	G	1			1	2	1	3
Weissella		**6**	**1**	**4**	**11 (11)**	**6**	**2**	**8 (13)**
W. minor	Ha-b	1		2	3	1		1
W. thailandensis	Ia-g	2		1	3	5	2	7
W. confusa	Ja-b	1			1			
W. cibaria	Ka-c	1		1	2			
W. soli	La-c	1	1		2			
Leuconostoc		**4**		**1**	**5 (5)**			
L. citreum	M	3			3			
L. lactis	Na-b	1		1	2			
Lactococcus				**11**	**11 (11)**		**8**	**8 (13)**
L. lactis	Oa-b			2	2			
L. garvieae	Pa-b			9	9		8	8
Pediococcus				**3**	**3 (3)**	**4**	**3**	**7 (12)**
P. pentosaceus	Qa-b			3	3	1		1
P. dextrinicus	Ra-c					3	3	6
Streptococcus						**3**		**3 (5)**
S. agalactiae	Sa-c					1		1
S. gallolyticus	T					2		2
Unknown species		**5**	**5**	**2**	**12 (12)**	**10**	**6**	**16 (27)**
Total		**34**	**32**	**34**	**100 (100)**	**34**	**26**	**60 (100)**

[a] ribotype, [b] vancomycin added to MRS broth, [c] psychrotrophs; MRS broth incubated at 6 °C, [d] plain MRS broth incubated at 25 °C

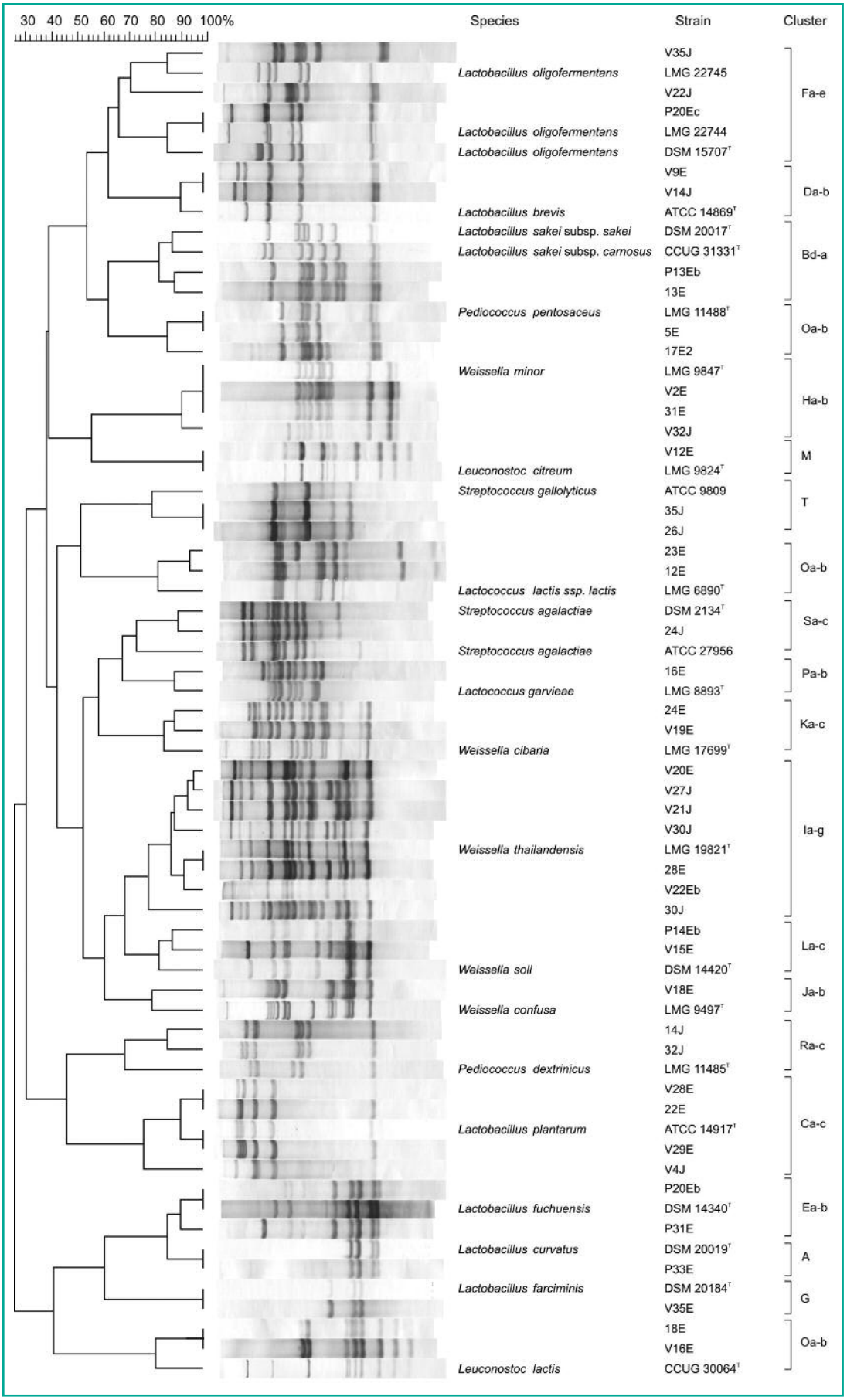

FIGURE 1: *Numerical analysis of HindIII ribopatterns showing all different ribotypes obtained from the LAB detected in skin samples and the patterns of the culture collection strains possessing the highest similarity to them.*

ted to *L. curvatus,* and was originally isolated in vacuum-packaged beef (Sakala et al., 2002), was likewise detected in the pre-scalding samples. The only psychrotrophic LAB detected both pre- and post-scalding was *L. oligofermentans.* This species has previously been associated with spoiled marinated MAP poultry (Koort et al., 2005).

Along with the meat lactobacilli, *Le. carnosum* and *Le. mesenteroides* have both been connected to spoiled cooked pork products, most recently artisanal ham (Vasilopoulos et al., 2008) and pork sausage (Laursen et al, 2009). *L. carnosum* has been reported to cause problems especially in vacuum-packaged cooked ham (Björkroth and Korkeala, 1997b; Björkroth et al., 1998; Samelis et al. 1998; Samelis et al., 2006; Vasilopoulos et al., 2008). Of leuconostocs, only *L. citreum* and *L. lactis* were detected in the pre-scalding skin samples and none in the post-scalding samples even the vancomycin enrichment was used for their selection. These two species have not been associated with cold-stored meat products. Our results suggest that the cutaneous microbiota of pig may not be rich with *L. carnosum* and carnobacteria. Similar result was obtained when cutaneous LAB associated with broiler chicken were studied by Vihavainen et al. (2007). LAB populations detected in broiler carcasses were neither containing leuconostocs nor carnobacteria whereas *L. sakei, L. brevis, Weissella paramesenteroides* and *pediococci* were detected. By contrast to the broiler carcasses, carnobacteria and leuconostocs were obtained from processing plant air.

In the present study and in the study of Vihavainen et al. (2007), *L. brevis* was associated with both pre- and posts-scalding samples. Of the LAB associated with meat fermentations, *L. plantarum, L. sakei, L. curvatus* and *P. pentosakeus* were detected, of which *L. plantarum* and *P. pentosakeus* also after scalding.

Pigs were clearly bringing a load of psychrotrophic LAB to the slaughterhouse even scalding, singeing and polishing of the carcasses had a clear diminishing effect on these LAB. Psychrotrophic LAB species are commonly detected in composting plant material, water and soil (Wood and Holzapfel, 1995) and can enter the processing facilities in a number of other ways too. However, our study shows that the meat-associated lactobacilli *L. curvatus, L. fuchuensis* and *L. sakei* are present on the pork skin and the animals may introduce these species to the slaughterhouse environment. This was not detected equally in case of meat-associated leuconostocs and carnobacteria. Their absence in the study suggests other sources of contamination in the meat production evironment.

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+++ Nachrichten aus Forschung und Industrie +++

(Die Verantwortlichkeit für die Texte liegt ausschließlich bei den Instituten und werbenden Unternehmen.)

Neuer ELISA-Test zur Detektion von Gliadin

Zöliakie ist eine Krankheit, die in den letzten 10 Jahren erhöhte Aufmerksamkeit erregt hat. Studien deuten darauf hin, dass in einigen Ländern bis zu 1 % der Bevölkerung eine Intoleranz gegen Gluten bestimmter Getreidearten aufweisen.

Diese Intoleranz basiert auf einer Stimulation des Immunsystems durch Gliadin, ein Gluten-Protein im Weizen, und entsprechender Proteine aus der Gruppe der Prolamin-Proteine, die in den Körnern anderer Triticeae-Arten vorkommen. Die meisten ELISA-Tests zum Nachweis von Gliadin sind Sandwich-ELISA die mit dem R5-Antikörper arbeiten.

Die Bindungsfähigkeit dieses Antikörpers sinkt deutlich bei hydrolisiertem Gliadin, das in vielen verarbeiteten Lebensmitteln vorkommt. Die TRANSIA GmbH, Ober-Mörlen bietet jetzt einen neuen ELISA-Test zur Detektion von Gliadin und entsprechender Proteine aus Gerste und Roggen an, den GLUTEN-TEC® ELISA.

Dieser kompetitive ELISA nutzt einen neuen monoklonalen Antikörper, der gegen die alpha-20-Peptidsequenz des Gliadins gerichtet ist. Diese Peptidsequenz ist direkt verantwortlich für die Stimulation der T-Zell-Immunantwort, und spielt damit die Hauptrolle bei der Pathogenese der Zöliakie. Der anti-alpha-20-Peptid-Antikörper ist unter der EU Patent-Nummer EP1779115 registriert und bindet gleichermaßen an natives und hydrolisiertes Gliadin.

Der GLUTEN-TEC® ELISA wurde in einem Ringversuch in 10 verschiedenen Laboren in Europa und den USA gemäß den Richtlinien der AOAC getestet. Dabei wurde gezeigt, dass mit dem GLUTENTEC® ELISA Gluten in rohen und erhitzten Lebensmitteln unterschiedlichster Art nachgewiesen werden kann. Getestet wurden unter anderem Mehl, Babynahrung, Fleischprodukte, Schokolade und Bier.

Die Nachweisgrenze (LOD) beträgt 2,5 ppm Gliadin, was 5 ppm Gesamt-Gluten entspricht. Der monoklonale anti-alpha-20-Peptid-Antikörper hat eine Spezifität von 100 % für das T-Zell-stimulierende Peptid des Gliadin und vergleichbarer Prolamine aus Roggen und Gerste.

Eine Kreuzreaktion mit Hafer, Mais, Reis, Hirse und Buchweizen wurde nicht festgestellt. Mit dem GLUTEN-TEC® ELISA geht TRANSIA GmbH einen weiteren Schritt, um die Sicherheit von Lebensmitteln durch sensitive und spezifische Analysen höchster Qualität zu verbessern.

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