Szent István University
Postgraduate School of Veterinary Science

Development and application of rapid microbiological methods in food and environmental hygiene examinations

Theses of PhD dissertation

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Introduction, aims of the study

The protection of public health is a fundamental aim of the food law. Microbiological hazards in the foodstuffs are the main source of the foodborne diseases. The food must not contain microbes, toxins, metabolites produced by the microorganism in such an amount which can mean risk for the human health.

The time requirement of the classical microbiological methods depending on the microbe is 1-4 days. .At the same time, the demand for faster assessment of feed lots, the efficient operation of the HACCP systems and the reduced duration of temporary storage require the hastening of microbiological evaluations and automation with potential simultaneous decrease of costs. In order to decrease the risk of human food borne infection and increase the effectiveness of the food chain control, much faster and automatable new techniques should be applied for the detection of pathogen microbes and for the qualitative and quantitative microbiological analyses of final product and product-in-process.

Results established by rapid microbiological methods can be fed back to the HACCP systems. Unsuitable products can be screened and inhibited their entering the food chain.

The MicroTester system developed and patented by the researchers of the Department of Food Hygiene (Szent István University, Faculty of Veterinary Science) and Department of Physics and Automatization (Corvinus University, Faculty of Food Science) is suitable for significantly reducing the time requirement of microbiological testing. The advantage of the redox-potential measurement method is to achieve the same productivity (sample number), but the investment cost is almost one third of that of the impedimetric methods and it allows the application of standard culture media.

The redox potential method is applied for water and environment hygiene examinations in waterworks' laboratories and bottling plants in Hungary and in several European countries.

The aim of my study is to prove the possibility of the application of the redox potential based measurement for examination of foodstuffs of animal origin (milk, meat) and environmental hygiene:

- Rapid determination of count of microbes important in process hygiene aspects by redox potential measurement:
  - Rapid determination of total count and Enterobacterium count in raw milk and meat
- Determination of microbial contamination of surfaces by redox potential measurement.
- Rapid detection of important microbes in aspects of food safety:

- Development and application of the combination of redox potential measurement and real-time PCR method for the detection of *Salmonella* and *Listeria monocytogenes.*

Materials and methods

The conventional cultural method was done according to following standards:
Total count determination (mesophil aerobe and facultative anaerobe microbes): MSZ EN ISO 4833:2003.
Detection of *Listeria monocytogenes:* MSZ EN ISO 11290-1:1998 (amendment: MSZ EN ISO 11290-1:1996/A1:2005).
Detection of *Salmonella*: MSZ EN ISO 6579:2006.
Microbiology of food and animal feeding stuffs. Horizontal methods for sampling techniques from surfaces using contact plates and swabs: MSZ ISO 18593:2008.
Determination of *Enterobacteriaceae* count: MSZ ISO 21528-2:2007.

The redox potential measurements were carried out by the MicroTester equipment, at the Food hygiene Department, Faculty of Veterinary Science.

The principle of the method is that in the measuring cell due to microbial multiplication, above a quite large (10^6-10^7 cfu/ml) critical microbe concentration the redox potential of the medium is well-detectably reduced and the rate of its change overcomes a defined detection criterion. The time to detection (TTD) means the time necessary to reach the detection criterion. There is a close linear correlation between TTD and the logarithm of the initial viable count. This relationship makes possible to determine the microbial count on the base of the time to detection.

Measurement based on internal calibration curve is applied when the composition of the microflora is not known and previously constructed calibration curve cannot be used. In this case, the redox potential measurement can be combined with the MPN method. From the sample the usual dilution range is prepared up to a dilution which already is without microbe. From each member of the dilution series 1 ml is put into a measuring cell and the redox curves are constructed. Based on the last dilution levels still showing multiplication, the most probable number of the initial living cells (MPN can be determined.
The measuring curve can be constructed from the lgMPN values and TTD values belonging to the certain dilution level, while the calibration curve demonstrates the lgMPN vs TTD connection.

Determination of total count and Enterobacteriaceae count

Raw milk samples

For the total count determination ½ Tryptone Soy Broth (TSB, Merck) at 30 °C, and for selective determination of Enterobacteria, Enterobacter Enrichment Broth (EEB, Merck) at 37 °C were used.

For internal calibration curve construction ½ concentration Tryptone Soy Broth (TSB, Merck) at 30 °C was used.

Stuffed meat products

10 g from the samples taken during stuffed meat product processing were homogenised in 90 ml peptone water then 1 ml was inoculated into 9 ml ½ TSB broth. For the construction of calibration curve tenfold dilution series was prepared. From each member of the dilution series 1 ml suspension was put into a measuring cell containing 9 ml of ½ TSB broth. Incubation temperature was 30 °C.
The calibration curves were constructed from the TTD values and microbe count determined by plating or MPN method.

Surface hygiene examinations

Applying the redox potential measurement the enumeration of the microorganisms wiped from the surface could be performed in two ways.

– From the dilution liquid after washing down the swab (similarly to the plate counting).
– Directly from the swab without any washing.

1/2 TSB broth was used for the determination of total count.
Sampling was carried out regarding to the ISO 18593:2004 standard: min. 10x10 cm sampling surface or in the case of smaller surfaces the whole surface was wiped. For swabbing of surfaces, "Eurotubo® collection swab" tampons were used. Hygiene samples representing of 100 cm^2 surfaces were taken at 10 points of a meat manufacturing pilot plant at the end of the daily processing.

Detection of *Listeria monocytogenes*

Selectivity examinations

The following bacterial strains were used: *Listeria monocytogenes* (ATCC 19111, ATCC 7644, NCAIM B1935), *Listeria ivanovii* (ATCC 19119), *Listeria innocua* (ATCC 33090), *Staphylococcus aureus* (ATCC 12600), *Escherichia coli* (ATCC 105369), *Bacillus cereus* (NCAIM B1827), *Bacillus subtilis* (NCAIM B1095).

For choosing the most selective culture medium, bacterial growth was monitored by redox potential measurements in three different types of media:

a) Listeria Enrichment Broth (LEB) Base acc. to FDA/IDF-FIL (Merck) with Oxford Listeria Selective Supplement (Merck)
b) Listeria Enrichment Broth (LEB) Base acc. to FDA/IDF-FIL (Merck) with Listeria Selective Enrichment Supplement acc. to FDA-BAM 1995/IDF-FIL (Merck)
c) Fraser Listeria Selective Enrichment Broth (Merck) with Fraser Listeria Supplement (Merck)

Examinations of food samples

25 g of soft cheese or 25 ml raw milk samples were homogenized in 225 ml of the Listeria Enrichment Broth (LEB) with Oxford Listeria Selective Supplement, which was the most selective for *L. monocytogenes* among the examined three media. Raw milk and soft cheese samples were obtained from the local market.

The food samples spiked with the test microorganisms were examined at three contamination levels (low, medium and high).

From each test microbe a 24- hour slant agar single culture was washed with 9 ml peptone water and a tenfold dilution series was prepared. The samples (25 g in 225 ml broth) were inoculated with 1 ml of the 9th, 6th and 1st dilution of the single culture to set the different contamination levels, respectively. The incubation temperature was 37 °C.

The actual microbial counts of the inocula were determined by plate counting from the dilution series in 3 parallels and referred to 1 ml or g of the sample.

Experiment design:

2 products (milk and soft cheese),

8 microbes (natural + 7 test microbes)

3 contamination levels (low, medium, high)

3 parallels

Sample size n = 144

The redox potential measurement, as an enrichment process, could screen the samples which do not contain *Listeria monocytogenes*. Only the presumably *Listeria monocytogenes*-positive samples need the expensive PCR identification.

Detection of *Salmonella*

The selectivity of RVS broth was examined by adding milk. 25 ml milk was homogenized in 225 ml RVS broth. Separated samples were spiked with *Proteus vulgaris*, *Klebsiella oxytoca*, *Escherichia coli*, *Citrobacter freundii*, *Salmonella* Enteritidis and *Salmonella* Typhimurium respectively.

Examination of food samples

The whole eggs were homogenised and then the egg samples were spiked with *Salmonella* Enteritidis and *Salmonella* Typhimurium. Three contamination levels (low, medium and high) were set for the microbes:

- low: 10^0 cfu/25g
- medium:10^2 cfu/25g
- high:10^4 cfu/25g.

25 g or ml food samples (egg, meat) were homogenized in 225 ml of RVS broth. The incubation temperature was 42°C.

During the enrichment phase the *Salmonella* positive samples could be screened by the redox potential measurement technique. Instead of biochemical confirmation, further identification was carried out by applying real-time PCR technique.

Real-time PCR

Genomic DNA was isolated from 1 ml of enriched food sample using "*Mericon* DNA Bacteria Plus Kit" (in case of *L. monocytogenes*) and *Mericon* DNA Bacteria Kit" (in case of *Salmonella*).according to the manufacturer's instructions. Real-time PCR amplification was performed in SLAN® Real-Time PCR System (Hongshi) using *Mericon L. monocytogenes* Kit (Qiagen) and *Mericon Salmonella* Kit (Qiagen) designed for the qualitative detection of *Listeria monocytogenes* and *Salmonella* in food and animal feed after enrichment. PCR was

done in final volume of 20 µl including 9.2 µl DNA isolate and 10.8 µl Multiplex PCR Mastermix. The cycling parameters consisted of an initial PCR activation step, activation of HotStarTaq Plus DNA Polymerase (5 min, 95°C), followed by 40 3-step-cycles consisting of denaturation (15 sec at 95°C), annealing for 23 sec at 60°C, and extension for 10 sec at 72°C. Fluorescence detection was performed at the end of the annealing stage of each cycle.

Results

Determination of total count and Enterobacterium count

By classical method, the total count determination of raw milk requires 72 hours. Although the microbe composition of the raw milks is different, the combined calibration curve constructed from independent samples resulted in strong linear correlation between the lgN_0 and TTD values. The origin of the milk samples were markets and dairy farms. The calibration curve is shown in Fig. 1. The time requirement of the total count determination by the redox potential measurement is 8-9 h in case of 10^4-10^5 cfu/ml microbe concentration, which is established by the dairy industry as microbiological criteria.

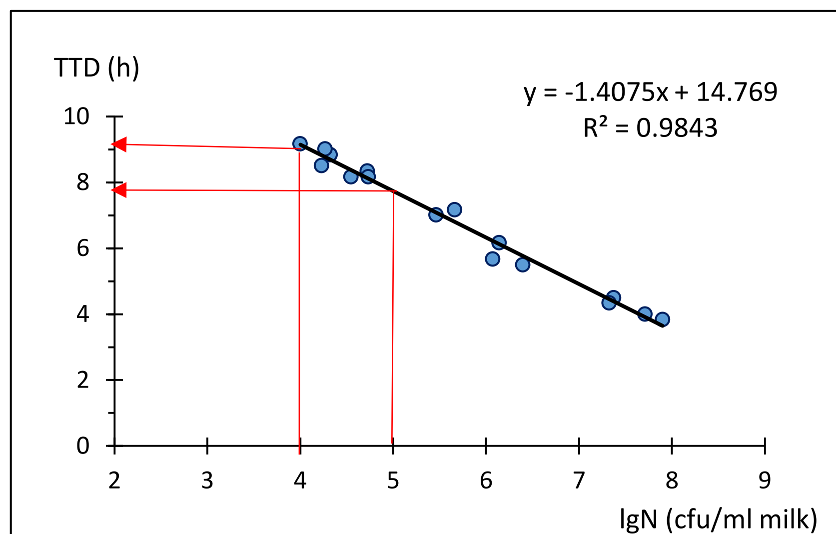


Fig.1. External measuring curve of total count in raw milk (1/2 TSB, T=30°C)

Total count and Enterobacterium count determination by internal calibration curve

This method is applied when the composition of the microflora is not known and previously constructed calibration curve cannot be used. In this case, the redox potential measurement could be combined with the MPN method based on the shape of the redox curves (Fig. 2.).

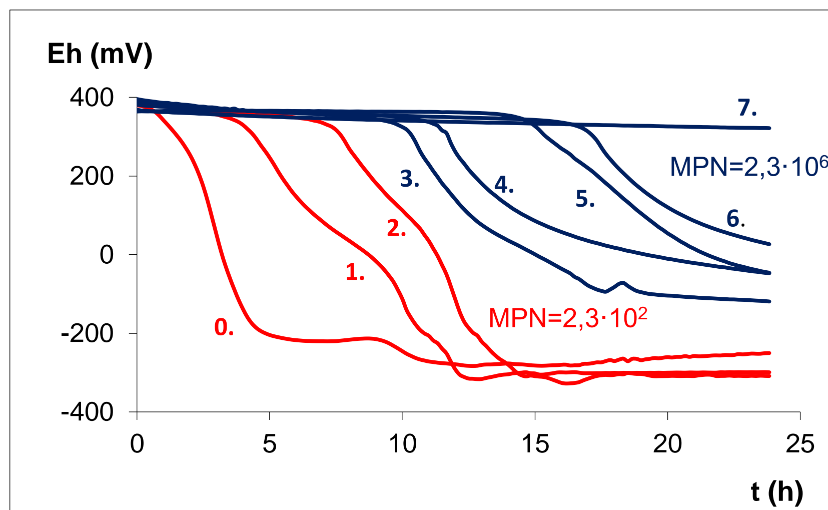


Fig.2. Redox curves of raw milk (1/2TSB, T=30°)

TTD value could not be detected in the 7th dilution. Total count was determined by MPN method: MPN=2,3x10^6 cell/ml.

Based on the MPN value and the TTD values belonging to the certain dilution levels, the internal calibration curve can be constructed.

The duration of the microbe count determination by MPN method is longer than it was with the external calibration curve but it is still significantly shorter than the usual 3 days of the culture method. A further advantage of the method is that due to the different shapes of their redox curves both the total count and Enterobacterial count can be determined simultaneously, applying non-selective nutrient broth (TSB) in a single, common measurement system. The background of this possibility is that from bacteria ordinary present in the food industry only the Enterobacteria are able to reduce the redox potential below -300 mV in aerobe culture media used in the redox potential measurements. Enterobacterium can be detected until the second dilution (signed with red). Enterobacterium count in the sample: 10^2 cell/ml.

The external and internal calibration curves of milk samples from various places did not show significant difference, common calibration curve can be constructed.

There is no significant difference between the total counts of raw milk determined by the plating and redox potential method, but the time requirement of the redox potential method is less.

Examination of stuffed meat products-in-process

Internal calibration should be applied because the composition of the microflora is not known

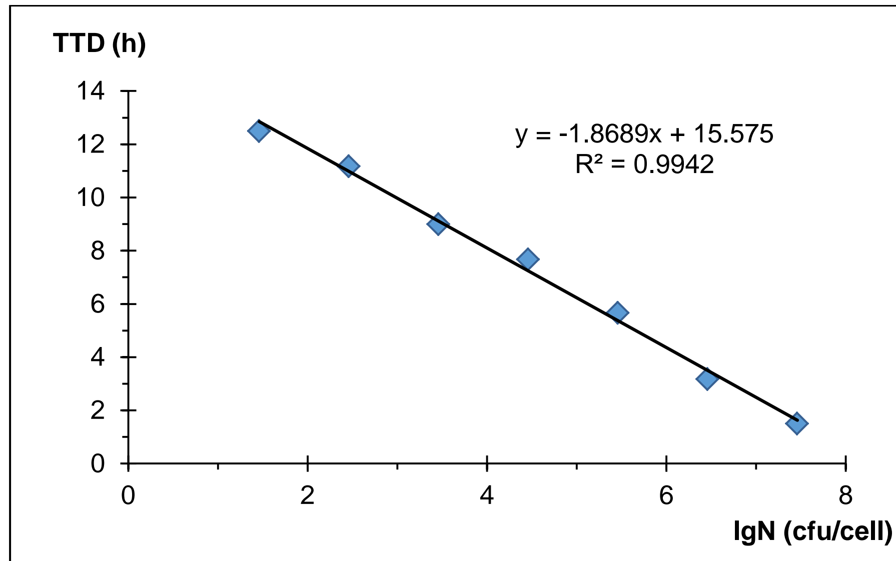
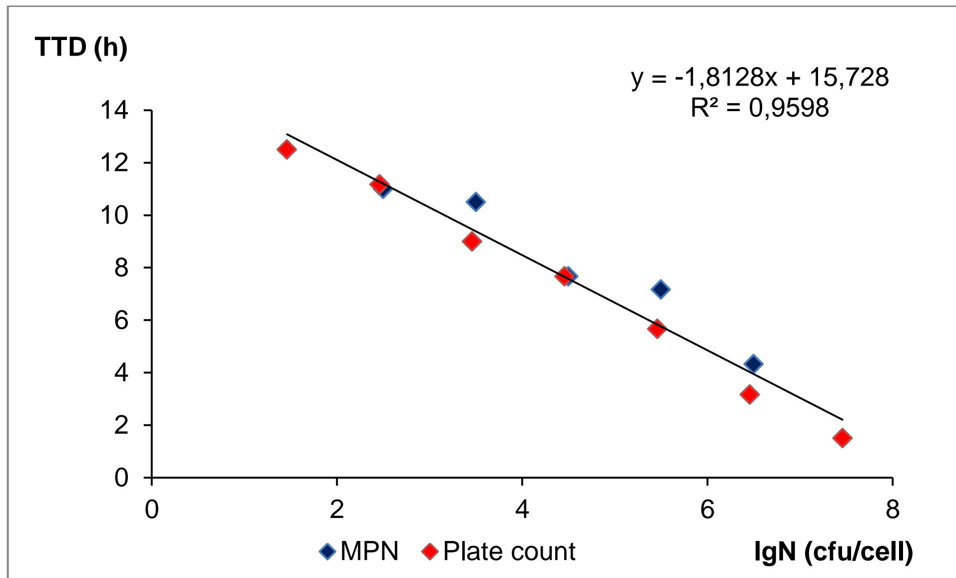


Fig.3. Measuring curve of frankfurter paste (1/2 TSB, T=30 °C)

Equation of calibration curve: $lgN = -0.532 \cdot TTD + 8.3114$. Based on calibration curve the total count of the samples taken at each phase point can be determined by the measured TTD values.

Determination of common calibration curve

Supposed that the composition of the microflora of certain phase points do not change, measuring curve taken at different time can be combined. The common measuring curve is shown in Fig 4..



Fig, 4. Common measuring curve of phase examinations

Equation of the calibration curve: lgN(cfu/cell) = -0.5294·TTD(h)+8.507

Samples were taken from certain phase points of a process to check the common calibration curve. Total count was determined by plating and redox potential measurement method too. The total count of the samples determined by the two methods is shown in Fig. 5.

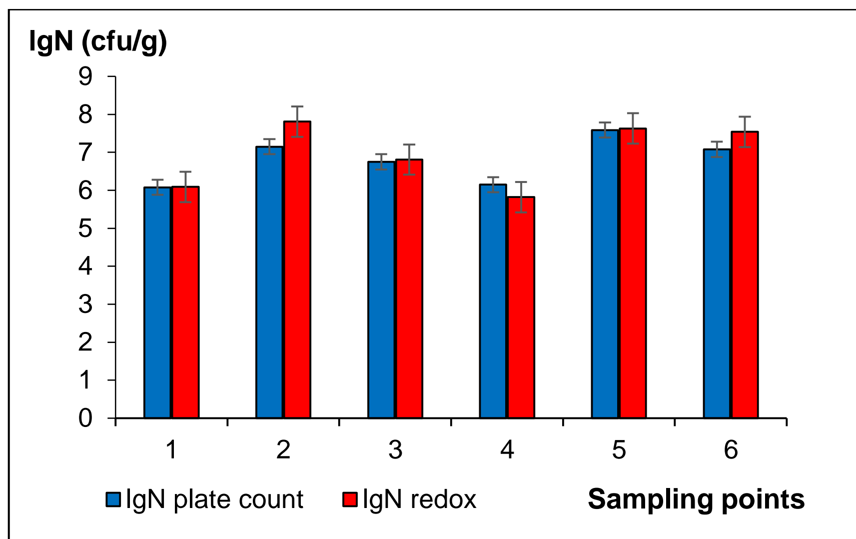


Fig.5. Total count determined by plating and redox potential measurement (lgN ± SD)

Microbial hygienic control by redox potential measurement

Calibration curve was constructed by the use of the internal measuring curves of chopper surface. The equation:

$$lgN = -0.2859 \cdot TTD + 7.2672 \qquad R^2 = 0.9574$$

Based on the calibration curve the total count of the surfaces was determined. The samples of all sampling points were examined by classical and redox potential method too.

Results of the classical and redox potential measurement with 95% confidence intervals are shown in Fig.6.

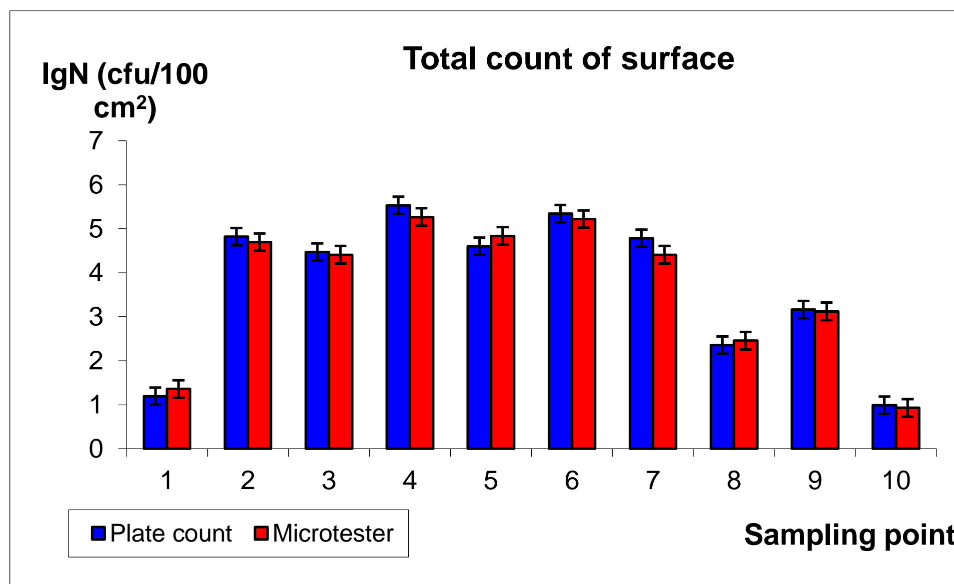


Fig.6. Total count of surface by classical and redox potential measurement

The evaluation of swab examinations by the application of redox-potential measurements can significantly be accelerated. By help of internal calibration curve, the viable count of surfaces with unknown microflora may also be determined. Using internal calibration and depending on the level of surface contamination, the viable count can be determined within 15–20 h. In further studies of surfaces in certain plant, the already established calibration curve may be applied as an external one. Using external calibration curve (depending on the level of the surface contamination) the viable count may be determined within 4–8 h. The time requirements of the classical and redox potential method are summarized in Table 1.

Table 1. Time requirements of the standard and redox potential measurement methods

lgN = cfu/100 cm^2	Detection time (h)	
	Standard	Redox
5.76	72	7
5.57		8.17
5.25		8.5
5.25		9
4.82		10
4.98		10
4.93		11.17
3.93		14.17
2.86		16.83

Advantages of the application of external calibration curve are that washing of swabs and the preparation of dilution series could be omitted. The duration of the examination, the material, tool and labour requirements can significantly be reduced.

Detection of *Listeria monocytogenes*

Bacterial growth was monitored by redox potential measurements in three different types of media at 37 °C. Each culture medium (250 ml) was inoculated with 1 ml (about 10^6 cfu) single culture of test microorganism.

LEB with Oxford Listeria Selective Supplement (Merck) showed the highest selectivity for the detection of *L. monocytogenes*, therefore it was used in the investigation of the prepared samples.
Growth: All of the three *L. monocytogenes* strains, *L. innocua, B. subtilis* No growth: *L. ivanovii, E. coli., B. cereus* Inhibited: *Staphylococcus aureus*.

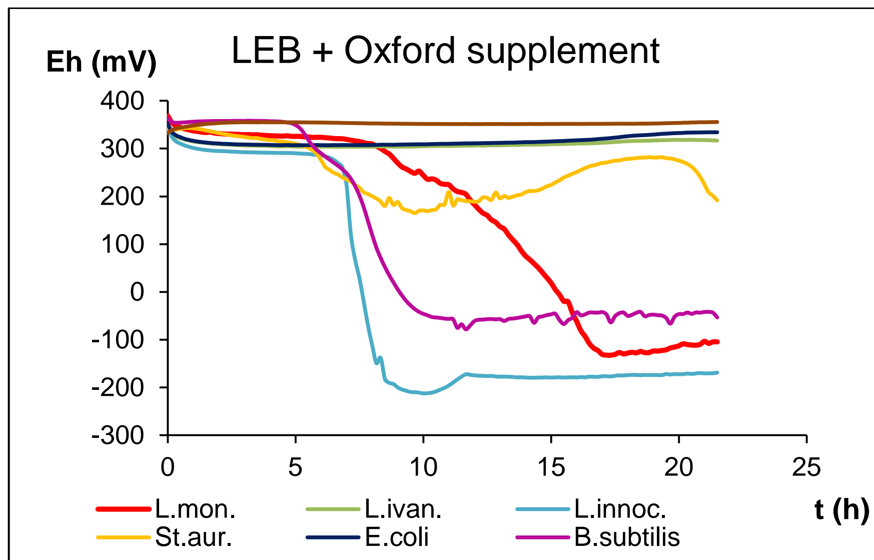


Fig. 7. Selectivity of LEB broth with Oxford supplement

Considering that the *E. coli* was totally inhibited and the partially inhibited *Staphylococcus aureus* did not reduce the redox potential of the medium to the level of *Listeria* spp., in the presence of *L. monocytogenes* or *L. innocua* the characteristic shape of *Listeria* redox-curves could be recognized. In presence of *Bacillus subtilis* the redox potential drops below the level of *Listeria* spp., so in this case the presence of *L. monocytogenes* couldn't be excluded. Based on the redox potential measurement the LEB with Oxford supplement was the most selective medium for *Listeria monocytogenes*, but the redox curves of the three *Listeria* spp. and *Bacillus subtilis* could not be differentiated. Due to this disability of differentiation, in each case when the presence of *L. monocytogenes* could not be excluded the identification had to be done. To confirm the presence of *L. monocytogenes* real-time PCR technique was used.

Determination of calibration curves of Listeria spp.

The redox potential measurements were performed in LEB with Oxford supplement. The measuring curve can be constructed from the lgN and TTD data pairs belonging to the same dilution levels.

The measurement of the three *L. monocytogenes* strains (ATCC 19111, ATCC 7644, NCAIM B1935) resulted in a common curve (Fig. 8).

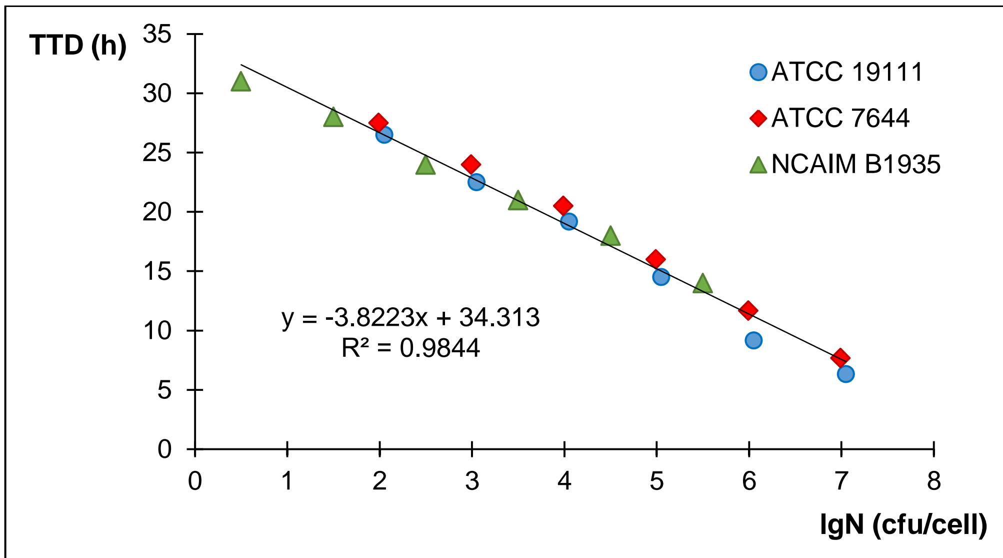


Fig. 8. Measuring curve of *L. monocytogenes* strains. in LEB broth with Oxford supplement

The equation of the calibration curve:

$$TTD = 34.31 - 3.8223 \cdot lgN$$

The theoretical time requirement of detection of one target microorganism in the measuring cell (lgN=0) can be calculated from the intercept of the measuring curve. TTD = 34.31 h.

The common figure representing the data of *Listeria monocytogenes* and *Listeria innocua* shows that the two measuring curves are significantly different (Fig. 9).

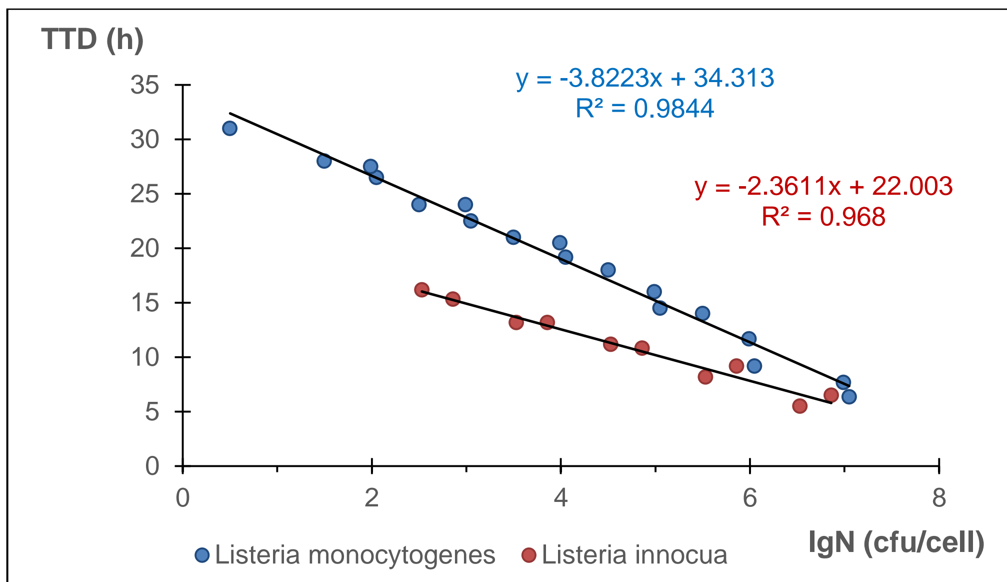


Fig. 9. Measuring curves of *Listeria monocytogenes* and *Listeria innocua* in LEB broth with Oxford supplement

Completing the calculation for the slower growing *L. monocytogenes* and taking into account the upper limit of the intercept (35.44 h), if we do not obtain TTD within 36 h, the inoculum of the measuring cell is free of *L. monocytogenes* with high probability. Getting a TTD value means a presumably positive sample. To confirm the presence of *L. monocytogenes* real-time PCR technique was used. To obtain TTD value indicated that the microbe concentration in the test cell reached about 10^6cfu/ml, which is higher than detection limit of the applied PCR technique lgN=10^3 microbe/sample ml, so the same suspension is ready for direct DNA isolation and real-time PCR assay.

Identification of L. monocytogenes from presumably positive samples by PCR

The identification from the enriched suspension by real-time PCR required another 3 hours. Due to the specificity of the Mericon *L. monocytogenes* Kit, only those samples found to be positive, which were spiked with *L. monocytogenes*. Samples, which - by redox potential measurement - were suspected to contain *L. monocytogenes*, but actually were spiked with *L. innocua* or *B. subtilis* found to be negative in case of each parallels of all dilutions. The results of the spiked samples at low contamination level (positive or negative), the time requirement of the redox potential measurements and real-time PCR, and their combination compared to the conventional ISO 11290-1 standard method are summarized in Table 2.

Table 2. Detection and identification of *L. monocytogenes* at low contamination level

Method	N	Standard	Redox potential		Real–time PCR		Total
	(cfu/ml)	growth		TTD (h)		time (h)	time(h)
Milk		- - -	- - -	36<		-	36
L. monocytogenes	$2.1 \cdot 10^0$	+ + +	+ + +	31	+ + +	3	34
L. ivanovii	$4.1 \cdot 10^0$	- - -	- - -	36<		-	36
L. innocua	$3.3 \cdot 10^0$	- - -	+ + +	20	- - -	3	23
Staph. aureus	$4.2 \cdot 10^0$	- - -	- - -	36<		-	36
E. coli	$5.6 \cdot 10^0$	- - -	- - -	36<		-	36
Bacillus cereus	$1.5 \cdot 10^0$	- - -	- - -	36<			36
Bacillus subtilis	$2.5 \cdot 10^0$	- - -	+ + +	25	- - -	3	28
Soft cheese		- - -	- - -	36<		-	36
L. monocytogenes	$9.5 \cdot 10^0$	+ + +	+ + +	29	+ + +	3	32
L. ivanovii	$3.6 \cdot 10^0$	- - -	- - -	36<		-	36
L. innocua	$5.7 \cdot 10^0$	- - -	+ + +	21	- - -	3	24
Staph. aureus	$6.3 \cdot 10^0$	- - -	- - -	36<		-	36
E. coli	$2.1 \cdot 10^0$	- - -	- - -	36<		-	36
Bacillus cereus	$2.1 \cdot 10^0$	- - -	- - -	36<		-	36
Bacillus subtilis	$3.1 \cdot 10^0$	- - -	+ + +	24	- - -	3	27

N: microbe concentration of the inoculum, mean of 3 parallels, CV(N) = ±15%
Total: Total time requirement of identification, Redox + PCR
Standard: ISO 11290-1
TTD: Time to Detection, SD(TTD) = 0.25 h

Detection *of Salmonella*

The growth of *Proteus vulgaris*, *Klebsiella oxytoca*, *Escherichia coli*, *Citrobacter freundii*, *Salmonella* Enteritidis, *Salmonella* Typhimurium were examined in milk sample using RVS broth.

Only the *Salmonella* serovars multiplied in the RVS broth, the other microbes did not grow. Based on the results, it can be established that the selectivity of the RVS did not decrease by adding food. Calibration curves of Salmonella serotypes could be divided into 4 groups. In each group, common measuring curve can be applied.

Salmonella serotypes with common calibration curves:

I. *S*. Bredeney, *S*. Kottbus, *S*. Senftenberg, *S*. Stanley, *S*. Tennessee*

II. *S*. Kentucky, *S*. Montevideo, *S*. Ohio, *S*. Saintpaul, *S*. Thompson, *S*. Typhimurium*

III. *S*. Cerro, *S*. Enteritidis*, *S*. Infantis, *S*. Livingstone

IV. *S*. Newport*

*measuring curves of the serotypes determined in RVS (Fig. 10)

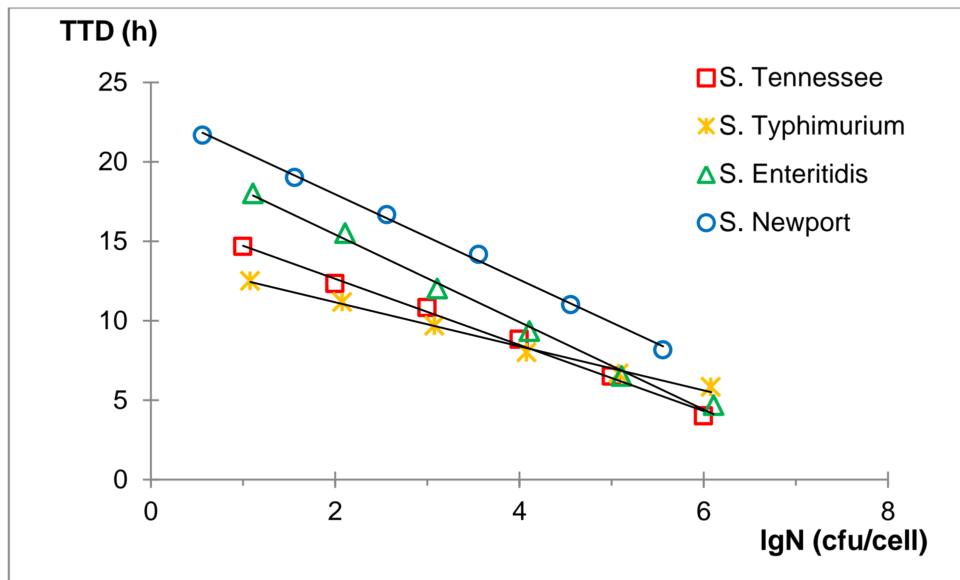


Fig.10. Measuring curves of *Salmonella* serotypes in RVS

Table 3. Equation of measuring curves of *Salmonella* serotypes (RVS broth)

Microbe	n	Equation	R^2	SD (h)
I. *S.* Tennessee	6	TTD(h) = 13.93 – 1.386·lgN	0.9926	0.250
II. *S.* Typhimurium	6	TTD(h) = 16.81 – 2.081·lgN	0.9947	0.319
III. *S.* Enteritidis	6	TTD(h) = 20.93 – 2.752·lgN	0.9938	0.453
IV. *S.* Newport	6	TTD(h) = 23.33 – 2.686·lgN	0.9976	0.278

The time for detecting one target microorganism (N=1) in the measuring cell can be determined from the intercept of the measuring curve (logN=0 The longest detection time belonged to *Salmonella* Newport in RVS broth, it was about 23.5 h

In practice therefore the duration of examination time could be determined based on the detection time of the slowest growing serotype of *Salmonella*. If during this time, which is needed for the detection of single cell of the slowest growing *Salmonella*, TTD cannot be detected, the food can be considered as *Salmonella*-free.

Detection of Salmonella *in artificially contaminated egg*

The results (positive or negative) and time requirement of the redox potential measurements and real-time PCR and their combination compared to the conventional ISO 6579 standard method are summarized in Table 4.

Table 4. Detection of *Salmonella* in egg (3 parallels)

Method	Standard		Redoxpotential		Real–time PCR		Redox+PCR
	result	time (h)	result	time(h)	result	time(h)	time (h)
natural microflora	-	66	-	24*	-	3**	27
low contamination							
S. Enteritidis	+	114	+	20.6[a]	+	3	23.6
S. Typhimurium	+	114	+	16.2[b]	+	3	19.2
medium contamination							
S. Enteritidis	+	114	+	16.7[a]	+	3	19.7
S. Typhimurium	+	114	+	11.7[b]	+	3	14.7
high contamination							
S. Enteritidis	+	114	+	13.2[a]	+	3	16.2
S. Typhimurium	+	114	+	8.7[b]	+	3	11.7
number of samples	21		21		21		21

*: no redox curve
**: no signal
[a]: SD=0,45 h,
[b]: SD=0,32 h

Salmonella *detection in broiler meat*

Salmonella contamination of broiler breast meat samples obtained from the local market was examined. All of the samples were examined also by the ISO 6579 conventional cultural method, parallel with the combination of redox potential based method and real-time PCR.

Table 5. Detection of *Salmonella* in broiler meat

	Standard		Redoxpotential measurement			Real-Time PCR		Total[a]
Sample	result	time	result	time	SD (h)	result	time	time
1.	+	114	+	11.7	0.123	+	3	14.7
2.	+	114	+	10.2	0.423	+	3	13.2
3.	+	114	+	15.8	0.113	+	3	18.8
4.	+	114	+	14.3	0.192	+	3	17.3
5.	+	114	+	13.6	0.280	+	3	16.6
6.	+	114	+	11.6	0.260	+	3	14.6
7.	+	114	+	9.2	0.323	+	3	12.2
8.	+	114	+	15.0	0.413	+	3	18.0
9.	-	66	-	24*		-	3**	27
10.	+	114	+	11.0	0.390	+	3	14.0
11.	+	114	+	11.8	0.303	+	3	14.8
12.	+	114	+	11.5	0.250	+	3	14.5
13.	+	114	+	13.0	0.250	+	3	16.0
14.	+	114	+	13.7	0.123	+	3	16.7
15.	+	114	+	12.3	0.333	+	3	15.3
16.	+	114	+	10.3	0.333	+	3	13.3
17.	+	114	+	8.8	0.250	+	3	11.8
18.	+	114	+	8.8	0.178	+	3	11.8
19.	+	114	+	15.2	0.373	+	3	18.2
20.	+	114	+	10.3	0.161	+	3	13.3

[a]: total = Redox + PCR method
* : no redox curve
**: no signal

During the enrichment phase the *Salmonella* positive samples could be screened by the redox potential measurement technique. Instead of biochemical confirmation, further identification was carried out by applying real-time PCR technique. In case of negative samples, the results could be obtained in 24 h by the redox potential measurement.

These negative samples were proved to be negative by the standard method also. In that case the PCR examination could be omitted.

Conclusions

Determination of total count and Enterobacterium count

Total count in raw milk can be determined by both external and internal calibration curve. Common calibration curve can be constructed from the data of the two methods.

There is no significant difference between the total counts determined by the plating and redox potential method. Measurement by the redox equipment is faster, especially with external calibration curve. In this case the determination of the critical total count of 10^4-10^5 cfu/ml in dairy industry requires about 8-9 hours.

Applying internal calibration curve, total count and Enterobacterium count can be determined simultaneously in a single, common measurement system. Comparing the time requirement of the methods, the traditional plating method demands 3 days for the determination of total count, while the redox method with internal calibration curve needs only 15–20 hours.

Redox potential method can be applied for the examination of meat and meat products.
The control of the microbial criteria established for the aerobe microbe count in the 2073/2005/EC with redox potential technique requires 6-10 hour in opposite to the prescribed 72 h incubation of the classical determination.

Microbial hygienic control

The evaluation of swab examinations by the application of redox-potential measurements can significantly be accelerated. The microflora present on the swab is directly measurable without washing and dilution. There is no statistically significant difference between the microbial counts obtained with redox-potential measurements and plating method.

The time requirement of redox potential technique, depending on the microbe count and the calibration curves, lasted 4-20 hours, in opposite to the standard 72 hours of the classical culture methods.

Detection of *Listeria monocytogenes*

In this study the possibility of the rapid and reliable detection of the bacterium in raw milk and soft cheese was demonstrated by combination of the redox potential measurement and real-time PCR methods. LEB with Oxford Listeria Selective Supplement (Merck) showed the highest selectivity for the detection of *L. monocytogenes*, therefore it was used in the investigation of the prepared samples.

During the enrichment phase the *L. monocytogenes* containing (*i.e.* presumably positive) samples could be screened by the redox potential measurement technique. Since the redox potential method was not able to distinguish the other *Listeria* spp. and *B. subtilis* from each other, the positive samples needed further identification by applying real-time PCR technique.

From the redox measuring curves it could be deducted that in case of the presence of *L. monocytogenes* the result is obtained in max. 36 h. If we get no TTD in 36 h, the sample is probably free of *L. monocytogenes* contamination. The proposed combination of the redox potential measurement and PCR technique was applied for detection of *L. monocytogenes* in raw milk and soft cheese samples artificially contaminated with Listeria and disturbing bacterium species. The investigations of the samples at three contamination levels showed no differences between the different spiking concentrations. Only the samples containing *L. monocytogenes* proved to be positive.

The results obtained by the combination of the two instrumental methods were totally identical with those of the classical nutrient methods. However, the time requirement of the investigations was significantly shorter. At low contamination levels (N<10 cfu/g,), below the detection limit of the direct PCR technique, the detection of *L. monocytogenes* required 34 h including the redox potential and PCR methods. At medium contamination level (10^3-10^4 cfu/g) the instrumental detection and identification needs about 24 h. At extreme high contamination (10^7-10^8 cfu/g) the time requirement is about 8h.

Comparing to the conventional culture method which requires 120 h for detection “no Listeria” or 168 h for “no *L. monocytogenes*” (ISO 11290-1, 1996), having the results by the proposed method couple in maximum 36 hours would provide a significant aid to the manufacturers to reliably determine the criterion that *L. monocytogenes* is not present in 25 g sample before the product leaves the production plant.

The redox potential measurement as enrichment process was able to screen the majority of the *L. monocytogenes*-negative samples. Only the presumably positive samples need the expensive PCR identification. The benefit of the combined method is that in case of *L. monocytogenes*-negative samples the cost of the PCR identification could be spared.

Salmonella detection

During the enrichment phase the *Salmonella* positive samples could be screened by the redox potential measurement technique. Instead of biochemical confirmation, further identification was carried out by applying real-time PCR technique. In case of negative samples, the results could be obtained in 24 h by the redox potential measurement. Compared to the 114 h detection time of the conventional culture method, having the results in maximum 27 hours would provide a significant aid to the manufacturers to reliably meet the criterion that *Salmonella* is not present in 25 g sample during the shelf-life of the product. The combination of the redox potential and PCR methods is labour-saving and it significantly decreases the cost and time of detection of *Salmonella*.

New scientific results

- I made the further development of the redox potential measurement to make the method suitable for milk and meat industry application in process hygiene, environmental hygiene and food safety examinations. I proved that microbial results served by the method are not significantly different from the ones of the standard method, but the time requirement (usually 1 day) is shorter than the classical 3 days of the nutrient methods.

- I adapted the redox potential measurement based method for the important process hygiene criteria, total count and Enterobacterium count, rapid (6-8 h), simple and cost effective determination. I experimentally proved the applicability of the method for microbiological examination of raw milk, meat and environmental samples. Results established by redox methods can be fed back in the HACCP systems.

- I developed the combination of redox potential measurement and real-time PCR method for the rapid detection of *L. monocytogenes* in food samples in labour-saving and cost-effective way. The redox potential measurement, as an enrichment process, can screen the samples which do not contain *Listeria monocytogenes*. Only the presumably *Listeria monocytogenes*-positive samples need the expensive PCR identification.

I developed the combination of the redox potential and PCR methods for rapid detection of *Salmonella* in food. The combined method is labour-saving and it significantly decreases the cost and time of detection of *Salmonella* to 1 day. During the redox potential measurement, as an enrichment phase, screened the *Salmonella* positive samples. Instead of biochemical confirmation, further identification was carried out by applying real-time PCR technique. In those cases when the redox potential measurement gave *Salmonella* negative results, the PCR identification could be omitted.

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