PathogenCombat
for safe food

Project no.: 007081

Project acronym: PathogenCombat

Project title: Control and prevention of emerging and future pathogens at cellular and molecular level throughout the food chain

Instrument: Integrated Project (IP)

Thematic Priority 5 – Food Quality and Safety

Deliverable 13.54
Transfer into practical application of Good Hygienic Practice for water supply and usage in SMEs

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Revision [Draft 1]

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	**X**
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

1 Statement

Deliverable completed.

2 Explanation and corrective action

This deliverable has already been completed.

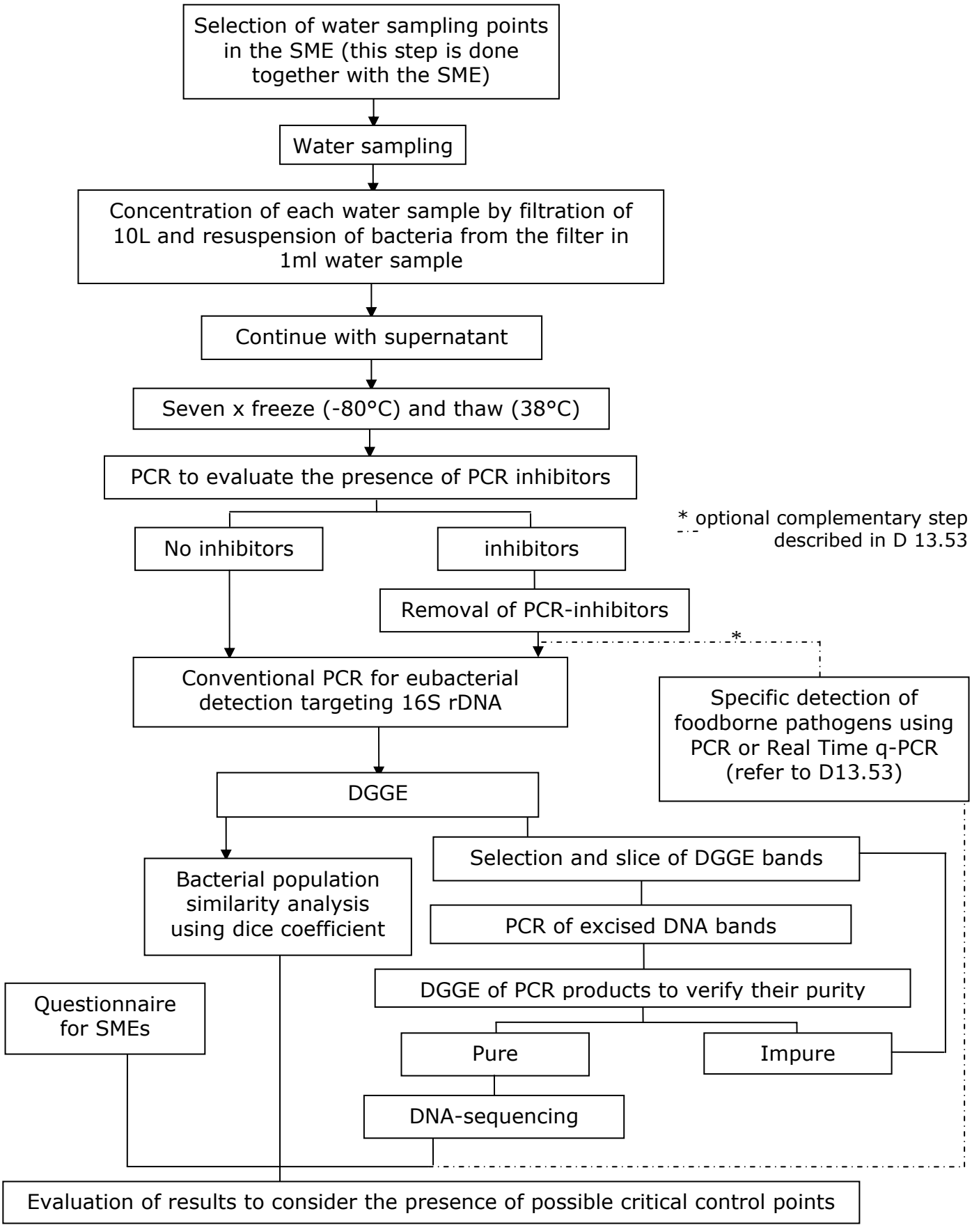
3 Use of Deliverable in PathogenCombat

The European Union food hygiene guidelines, according to the principles of the Hazard Analysis and Critical Control Points (HACCP), stipulate the introduction of self-control systems for food industries. The HACCP concept confers an important contribution to consumer's health protection by controlling the production, treatment, processing, transport, storage and sale of food. According to the international definition this concept aims to identify important possible health threaten dangers, these dangers are then analyzed, their occurrence are determined and their importance for health is measured. Finally, critical control points during the food production process are specified, steps in the process that could carry a danger are avoided or reduced to an acceptable level. In addition, each country has to follow the drinking water guidelines stipulated by their own authorities in order to have a good water quality.

The aim of this deliverable in the project is to apply new culture-independent methods to look for possible water-derived critical control points in the production lines of food industries. This is achieved by a integral evaluation of the water analysis of each SME, based principally in the use of DGGE (described below), and complemented with the specific detection of pathogens (methods are described in D13.53 and results are described in D13.55), DNA sequencing, interpretation of the questionnaire for SMEs concerning their drinking water distribution (see below) and a mutual work together and discussion with the SME.

DGGE is a molecular technique used as a tool to analyze the autochthonous bacterial population stability of bulk water at different sampling points of the SMEs. In that concern, unknown technical or material derived impacts on drinking water population become visible indicating a possible critical control point during food production. Selected DGGE DNA bands can be sequenced, and the nucleotide sequences can be aligned with ribosomal databanks.

The following flow chart describes the developed strategy used for the detection of possible water-derived critical control points at the SMEs. This flow chart could be further applied in WP 15 in order to develop a generic Food Safety Management System (FSMS).


Selection of water sampling points in the SME (this step is done together with the SME)
Water sampling
Concentration of each water sample by filtration of 10L and resuspension of bacteria from the filter in 1ml water sample
Continue with supernatant
Seven x freeze (-80°C) and thaw (38°C)
PCR to evaluate the presence of PCR inhibitors
No inhibitors
inhibitors
* optional complementary step described in D 13.53
Removal of PCR-inhibitors
*
Conventional PCR for eubacterial detection targeting 16S rDNA
Specific detection of foodborne pathogens using PCR or Real Time q-PCR (refer to D13.53)
DGGE
Bacterial population similarity analysis using dice coefficient
Selection and slice of DGGE bands
PCR of excised DNA bands
DGGE of PCR products to verify their purity
Questionnaire for SMEs
Pure
Impure
DNA-sequencing
Evaluation of results to consider the presence of possible critical control points

PROTOCOL FOR THE IDENTIFICATION OF POSSIBLE WATER-INVOLVED CRITICAL CONTROL POINTS AT FOOD INDUSTRIES

For the detection of possible water-derived critical control points, it is necessary to work in cooperation with the SME. A questionnaire over the SMEs drinking water sources, conditioning and distribution during food processing, should be filled up by the SME. This questionnaire will be used as a complementary tool for a better understanding of the laboratory results.

3.1 Water sample preparation for culture independent methods

Together with the SMEs several sampling points are selected in order to do an autochthonous bacterial population analysis. Water samples are aseptically taken at each selected sampling point using sterile flasks and are cool-transported as fast as possible for laboratory analysis. If the water cannot be processed in the same day it should be kept at 4°C.

Sample volumes were adapted to the detection limits of the methods (see section 3.1.2 and 3.4 in D13.53). This strategy was chosen to avoid any culture dependent pre-enrichment and pre-selection of pathogens.

The detection of PCR inhibitors and prevention of PCR inhibition was examined before running the pathogen and eubacterial specific PCR experiments (see section 3.1.2 in D13.53).

Methods for autochthonous bacterial population analysis (PCR-DGGE) are described in section 3.2. in D13.53

Recommendations to the SMEs Concerning Drinking Water Distribution

A) Origin and Processing of Drinking water

The quality of the supplied drinking water is one critical point for good hygienic practice in downstream process lines. Therefore, information about raw water qualities (surface water or groundwater) is needed in concern of potential contaminations with hygienically relevant bacteria. Subsequently, data about the drinking water conditioning at the waterworks are essential for the estimation of the biological stability of the drinking water during distribution. In case of the use of surface water a subsoil passage (embankment filtration) result in a pre-purification of raw water upstream of the waterworks. Different filtration steps (sand filtration, granular activated carbon filtration) remove unwanted organic matter, which might also influence microbial growth in downstream distribution systems. Disinfection measures are most important for the inactivation of micro-organisms. Chemical (chlorine, chlorine dioxide, ozone) and UV irradiation are the most frequently used disinfection techniques at European waterworks. The efficiency of the disinfection depends on the water parameters like turbidity,

organic matter, composition of bulk water microflora. Depending on the drinking water character the sustainability of the disinfection measure is impaired. The detection of indicator micro-organisms for irregular operation or faecal contaminations (e.g. *E. coli*, enterococci) is routinely performed with conventional plating methods. Drinking water authorities supervise the regulations for the drinking water production and distribution.

The SMEs, in consequence, should reflect these basic data from the public drinking water supplier to assess potential contaminations during food production. Some points are most critical: the discharge of the raw water with hygienically relevant bacteria and organic matter, and the efficiency and sustainability of the used disinfection technique at the waterworks.

B) Drinking water distribution at the SME

Secondary treatment of drinking water (e.g., softening etc.), pipeline materials, age of the pipelines, and the connections of the pipelines are parameters that might influence the growth (or re-growth) of micro-organisms in the SMEs drinking water distribution system. Biofilms, which are adherent bacteria embedded in a matrix of secreted polymeric substances (polysaccharide, proteins, DNA etc.), are potential habitats for all kind of bacteria including pathogens, and may be responsible for contaminations of the bulk water systems. Incrustations in e.g. old pipes, resin characters of softening facilities, the use of not certificated materials, and connection points of different kind of materials support the biofilm formation.

The SMEs, in consequence, should reflect these points and should control their drinking water distribution pipelines within their company to avoid the use of different materials or stagnation of drinking water, which might contribute to bacterial growth. The renew of drinking water pipelines as well as the occurrence of any damage of water pipelines (ruptures, corrosions) should be documented for potential correlations with bacterial contaminations. Additional local disinfection measures are requested for such critical points of the drinking water distribution.

C) Additional Aspects of Drinking Water Distribution

Accessory facilities like hoses used in cleaning processes during food production could be responsible for the occurrence and spread of hygienically relevant bacteria (cross-contamination). Biofilm formation at the inner surfaces of hose materials serve again as a potential contamination sources. Therefore, it is important to control such facilities in concern of biofilm formation. Such hoses should be exchanged in a regular manner, especially when warm water is used.

As warm water systems support the growth of bacteria, especially hygienically relevant bacteria (*E.coli, Pseudomonas aeruginosa, Legionella*

spp.) the warm water facilities should be controlled for the presence of such micro-organisms. Regulation for the operation of enlarged warm water production facilities are available and should be implemented.

Furthermore, the dispersion of water at local positions during food production is a critical point for the dissemination of bacteria. A good hygienic practice and monitoring should be realized at such production points.

Questionnaire for SMEs Concerning Drinking Water Distribution

Origin and Processing of Drinking Water

1. Which waterworks supply you with drinking water?
2. Which types of raw water are used by the waterworks and how is the raw water processed?
3. Which disinfection measures are taken in drinking water processing?

Drinking Water Distribution

4. Does your company carry out a secondary treatment of the drinking water (e.g. additional disinfection, softening, etc.)?
5. Which materials were used for the drinking water pipelines and how old are the pipelines and connections?
6. Did you use several materials and in which order?
7. Did you renew your drinking water pipelines while using the building?
8. Did you detect any damage of the water pipelines in the last years (e.g. pipe ruptures, corrosion, etc.)?
9. Is the microbiological control of your drinking water carried out internally or externally? At which intervals?
10. Do you have a current version of a plan of all drinking water pipelines and flow directions of the drinking water in your production buildings?
11. What do you think is critical to drinking water hygiene?

Additional Aspects of Drinking Water Distribution

12. Which hose materials are possibly connected to the water pipelines and used for cleaning or food processing?
13. Are you able to provide information on your water consumption and allocate the amounts of water consumed to the production lines?
14. Do you also use warm water in production?
15. How is the warm water prepared and fed into the production line?
16. Do you spray or atomize water during production?
17. Is an emulsion prepared during production?

4 Verification of Deliverable

4.1 Materials and methods

Water samples were taken at the following food industries:

A) German dairy industry – **Partner31 -** (22.06.07) First sampling period.

B) German poultry industry – **Partner33** - (12.09.07)

C) Spanish poultry industry – **Partner29** - (02.06.08)

D) Spanish dry cured ham industry – **Partner34** - (05.06.08)

E) German dairy industry – **Partner31 -** (06.11.08) Second sampling period.

The methodology was optimized during the project reflecting the specific conditions of the German and Spanish SMEs. The following table summarizes the sampling procedures applied to the different SMEs.

Sampling / Procedure	Water				
	A	B	C	D	E
Sample concentration by filtration	x	x	x	x	x
Freeze-Thaw cell disruption	x	x	x	x	x
Exploration of PCR inhibitors			x	x	x
PCR inhibitors removal (PVPP)			x	x	
Genome amplification (REPLI-g)	x	x			
Bacterial Population analysis	x	x	x	x	x

Water samples taken at different production points were concentrated by filtration using mixed cellulose ester membrane filters (Whatman, Germany). Due to the low number of bacteria expected in drinking water samples, cells in sample were disrupted by the commonly used freezing-thaw method, and kept at -20°C until use.

The following table shows the concentration rates of the sampled drinking water to be used for the analysis.

	Water sample				
Sampling	A	B	C	D	E
Concentration rate of sample	X 2000	X 2666	X 3666	X 3666	X 10000

The protocol named above describes the steps that were followed to do the autochthonous bacterial population analysis. A complementary specific pathogen detection in drinking water was done using culture-independent techniques as described in D13.53.

Drinking water samples have a very low bacterial charge, whole genome amplification of the bacteria present in the samples using REPLI-g Kit (Qiagen, Germany) was done in the first two samplings (A and B). No significant improvement was achieved in concern of amplification efficiencies. Therefore, this enzymatic step for genome amplification was left aside during subsequent analyses of drinking water samples.

4.2 Results

As already described above, the amounts of water filtered in each SME were different depending on the previously examined protocols.

Different variants of DGGE (primer set up, use of REPLI-g, gel composition, DNA extraction, DNA amplification etc.) were performed in order to optimize the molecular analyses targeting 16S ribosomal genes in *Eubacteria*. Not all experimental results are shown; only the results based on the optimized protocol and data obtained for population comparison using the Dice Coefficient calculation are exposed.

The summary of the data obtained from the DGGE after the use of the Dice Coefficient is shown in tables. Sampling points are exemplarily shown for the German dairy SME. In all experiments the main entrance of controlled public drinking water to the food industry facilities is used as reference for our studies looking for possible Critical Control Points (CCPs) at the German SMEs, and in extension to the recommended work for the Spanish SMEs.

4.2.1 German dairy industry

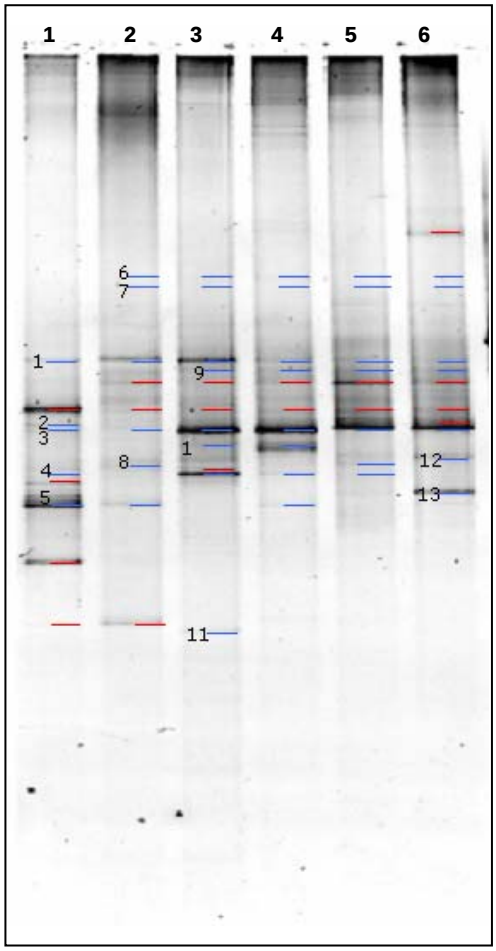
First Sampling period

Water samples were taken at six different points from the industry where water is used in direct or indirect food processing.

1. Entrance of controlled public drinking water
2. Lactic acid tank
3. Portioner
4. Hand washbasin
5. Maturation room
6. Feta packaging

The following DGGE gel picture shows the DNA fingerprints of the different sampling points at the German dairy SME. Representative DNA bands were excised and sequenced for bacteria identification (see numbers in the gel). The sequencing results from database alignments are listed in the table beside.

Primers used for PCR-DGGE: **27F-517R**



1	Uncultured bacteria	ß-proteobacteria
2	Acidovorax	
3	Uncultured bacteria	ß-proteobacteria
4	Uncultured bacteria	ß-proteobacteria
5	Caulobacter crescentis	α-proteobacteria
6	Acuabacterium	ß-proteobacteria
7	Acuabacterium	ß-proteobacteria
8	Sphingomonas	α-proteobacteria
9	Acinetobacter	γ-proteobacteria
10	Acuabacterium	ß-proteobacteria
11	Meiothermus	
12	Sphingomonas	α-proteobacteria
13	Sphingomonas	α-proteobacteria

Sampling point of dairy industry	Total DNA bands	Sequenced bands	Bacterial classes (number of sequenced bands)		
			α-Proteobacteria	*ß-Proteobacteria*	*γ-Proteobacteria*
1. Entry of public water	9	56	**1**	**3**	**-**
2. Lactic-acid tank	9	6	**2**	**4**	**-**
3. Portioner	11	8	**-**	**6**	**1**
4. Hand wash-basin	10	8	**1**	**6**	**1**
5. Maturation room	10	6	**-**	**5**	**2**
6. Feta packaging	12	7	**2**	**4**	**1**
Total Sequenced Bands		**13**			

Most of the identified bacteria belong to *β-Proteobacteria* subclass.

The number of total DNA bands was slightly increased in the water from the Feta packaging sampling point. This sampling point was attracted to attention when the bacterial population were compared in DGGE analyses, and identified as possible critical control point.

None of the targeted pathogens were identified when sequencing of DGGE bands was done, but some opportunistic bacteria were aligned: *Sphingomonas*, *Acinetobacter baumanii*.

The following table demonstrates the calculated Dice coefficient (Cs) and the percentage of similarity between two water samples (entrance point and one sampling point).

	b	**j**	Dice coefficient (**Cs**)	Similarity
A=**B**=Sample point 1	9	9	1	100%
B=Sample point 2	9	5	0,56	56%
B=Sample point 3	11	4	0,40	40%
B=Sample point 4	10	5	0,53	53%
B=Sample point 5	10	4	0,42	42%
B=Sample point 6	12	3	0,29	29%

A= Sample point 1
a= 9

Dice Coefficient: $\mathbf{Cs=2j(a+b)^{-1}}$, were **j** is the number of bands common to samples **A** and **B**, and **a** and **b** are the total number of bands in sample **A** and **B**, respectively. This index ranges from 0 (no common bands) to 1 (100% similarity of band patterns).

Additional information gathered from D13.53 and D13.55:

Pathogen detection using culture-dependent methods: No target pathogens (*E.coli, P.aeruginosa, C.jejuni, enterococci, and Salmonella*) were found after the use of culture dependent methods.

Pathogen detection using culture-independent methods: DNA from *C.jejuni* (point 1), enterococci (Point 2 and 6) and *P.aeruginosa* (Point 2) were found in some water samples, but the signals obtained were close to the detection limits of the assays or the amplification curves were irregular compared to positive controls.

Only one sampling point (Point 6. Feta packaging) was found to exhibit a similarity value below the threshold value of 40%, which is discussed as a possible critical control point.

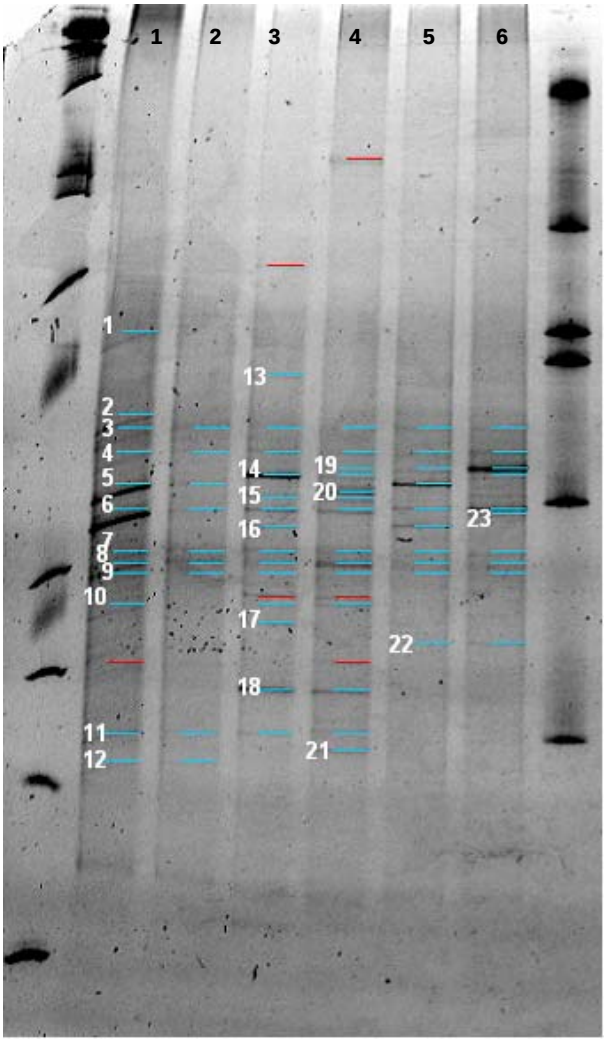
During the evaluation of the SME and during the discussion with the German SME no technical problems or irregular operation during food production became obvious. A correlation between the molecular biology detection of pathogens and extended populations shifts during PCR-DGGE was not always observed. Nevertheless we recommended changing hoses in higher frequency. A second sampling period was requested in order to use the optimized protocol, and compare the results obtained.

Second sampling period

Water samples were taken at the same points as in the first sampling period. The second sampling period was done in order to use the optimized protocol and to corroborate if the practical application of hygienic recommendations has a influence in the results of the autochthonous bacterial population analysis.

The following DGGE gel picture shows the DNA fingerprints of the different sampling points at the German dairy SME. Representative DNA bands were excised and sequenced for bacteria identification (see numbers in the gel). The sequencing results from database alignments are listed in the table beside.

Primers used for PCR-DGGE: **27F-517R**



1	γ-proteobacteria	γ-proteobacteria
2	Acidovorax sp.	ß-proteobacteria
3	Brevudimonas sp	α-proteobacteria
4	Uncult. ß-proteobacteria	ß-proteobacteria
5	uncultured	environmental
6	Iron-reducing bacteria	environmental
7	ß-proteobacteria	ß-proteobacteria
8	Uncult. δ-proteobacteria	δ-proteobacteria
9	uncultured	environmental
10	Uncult. ß-proteobacteria	ß-proteobacteria
11	Uncult. Chloroflexi	environmental
12	Uncult. Chloroflexi	environmental
13	Uncult. Soil bacteria	environmental
14	Uncult.Comamonadeceae	ß-proteobacteria
15	Uncult. γ-proteobacteria	γ-proteobacteria
16	(27)Methylibium petroleiphilum	ß-proteobacteria
17	(19) γ-proteobacteria	γ-proteobacteria
18	(20)Meiothermus timidus	Deinococcus
19	(25)Sphingobium sp.	α-proteobacteria
20	(22) Uncult. Sphingomonas	α-proteobacteria
21	(24) Uncult. γ-proteobacteria	γ-proteobacteria
22	(31) Uncult. ß-proteobacteria	ß-proteobacteria
23	(30) mixed	

Sampling point of dairy industry	Total DNA bands	Sequenced bands	Bacterial classes (number of sequenced bands)				
			α-Proteobacteria	*ß-Proteobacteria*	*γ-Proteobacteria*	*δ-Proteobacteria*	other
1. Entry of public water	13	12	**1**	**4**	**1**	**1**	**6**
2. Lactic-acid tank	9	9	**1**	**2**	**-**	**1**	**5**
3. Portioner	16	14	**1**	**5**	**2**	**1**	**7**
4. Hand wash-basin	17	14	**3**	**4**	**2**	**1**	**7**
5. Maturation room	10	10	**2**	**4**	**-**	**1**	**3**
6. Feta packaging	10	10	**2**	**4**	**-**	**1**	**3**
Total Sequenced Bands		**23**					

Most of the identified bacteria are uncultured environmental bacteria or belong to some *Proteobacteria* subclass, specially to the ß- *Proteobacteria.*

None of the targeted pathogens were identified when sequencing of DGGE bands was done.

The following table demonstrates the calculated Dice coefficient (Cs) and the percentage of similarity between two water samples (entrance point and one sampling point).

	b	**j**	Dice coefficient (**Cs**)	Similarity
A=**B**=Sample point 1	13	13	1,00	100%
B=Sample point 2	9	9	0,82	82%
B=Sample point 3	16	8	0,55	55%
B=Sample point 4	17	8	0,53	53%
B=Sample point 5	10	7	0,61	61%
B=Sample point 6	10	6	0,55	55%

A= Sample point 1
a= 13

Dice Coefficient: $\mathbf{Cs=2j(a+b)^{-1}}$, were **j** is the number of bands common to samples **A** and **B**, and **a** and **b** are the total number of bands in sample **A** and **B**, respectively. This index ranges from 0 (no common bands) to 1 (100% similarity of band patterns).

Additional information gathered from D13.53 and D13.55:

Pathogen detection using culture-dependent methods: No target pathogens (*E.coli, P.aeruginosa, C.jejuni, enterococci, and Salmonella*) were found after the use of culture dependent methods.

Pathogen detection using culture-independent methods: No target pathogens (*E.coli, P.aeruginosa, C.jejuni, enterococci, and Salmonella*) were found after the use of culture independent methods.

During the evaluation of the SME and during the discussion with the German SME no technical problems or irregular operation during food production became obvious. After the first sampling period we recommended changing hoses in higher frequency. A second sampling period was requested in order to use the optimized protocol, and compare the results obtained.

When the bacterial population of the water samples were compared with the entry water, at the second sampling period, no sampling point was found to be a possible critical control point, indicating that the drinking water in the entire industry is biologically stable.

These results confirm that the PCR-DGGE technique is reliable for the analysis and monitoring of the drinking water stability in different points at a food industry being a trustful tool for the seek of possible water-derived critical control points. This indicates that the aim of applying new culture-independent methods to look for possible water-derived critical control points in the production lines of food industries has been successfully fulfilled.

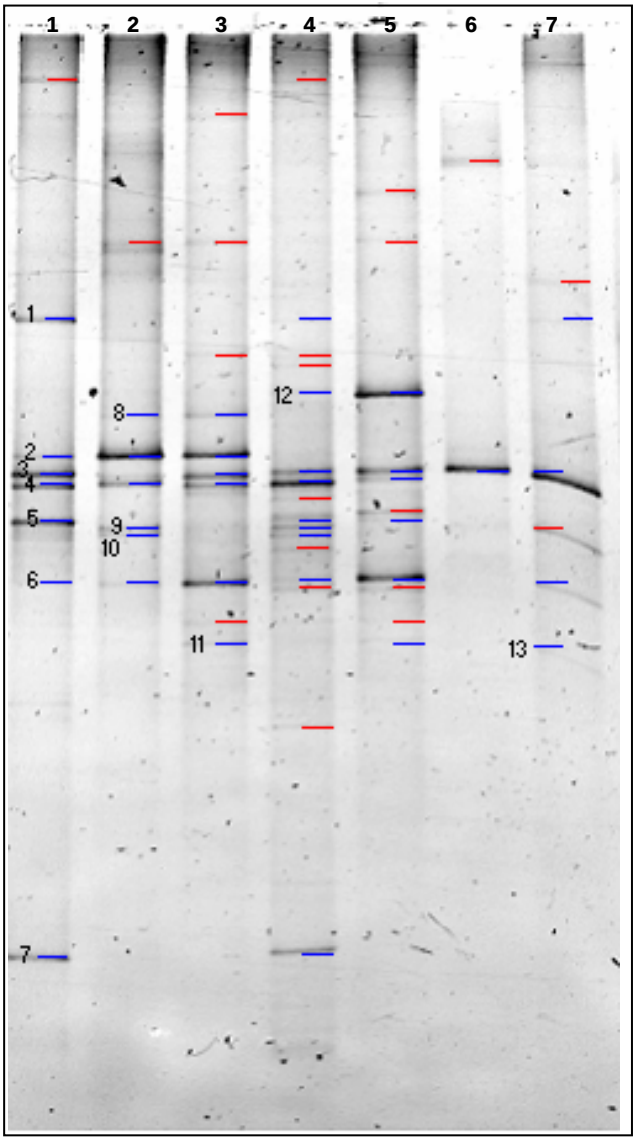
It is concluded that a good hygienic practice for water supply and usage are done at this SME, and no additional recommendations are required for this SME to maintain its high water quality.

4.2.2 German poultry SME

Water samples were taken at seven different points from the industry where water is used in direct or indirect food processing.

1. Income of state water
2. Slaughter room
3. Sausage production room
4. Preparation room
5. Preparation sink
6. Hygienic sluice
7. Slaughter sink

Primers used for PCR-DGGE: **27F-517R**



This DGGE gel picture shows the DNA fingerprints of the different sampling points at the German poultry SME. Representative DNA bands were excised and sequenced for bacteria identification (see numbers in the gel). The sequencing results from database alignments are listed in the table below.

1	Sphingomonas	α-proteobacteria
2	Comamonadaceae	ß-proteobacteria
3	Sphingomonas	α-proteobacteria
4	Acidovorax	ß-proteobacteria
5	Sphingomonas	α-proteobacteria
6	Caulobacter	α-proteobacteria
7	Thermus	
8	Acidovorax	ß-proteobacteria
9	Burkholderiales	ß-proteobacteria
10	Burkholderiales	ß-proteobacteria
11	Caulobacter	α-proteobacteria
12	Comamonas	ß-proteobacteria
13	Burkholderiales	ß-proteobacteria

Sampling point of poultry industry	Total DNA bands	Sequenced bands	Bacterial classes (number of sequenced bands)	
			α-Proteobacteria	*ß-Proteobacteria*
1. Entry of public water	8	7	**4**	**2**
2. Slaughter room	7	6	**1**	**5**
3.Sausage production room	10	6	**3**	**3**
4. Preparation room	16	9	**4**	**4**
5. Preparation sink	11	6	**4**	**2**
6. Hygienic sluice	2	1	**1**	**-**
7. Slaughter sink	6	4	**3**	**1**
Total Sequenced Bands		**13**		

Most of the sequenced DNA belongs to the *α-Proteobacteria* and *ß-Proteobacteria* subclasses.

None of the targeted pathogens were identified; only one opportunistic bacterium was aligned: *Sphingomonas.*

	b	j	Dice coefficient (**Cs**)	Similarity between A and B
A=B=Sample point 1	8	8	1	100%
B=Sample point 2	7	3	0,40	40%
B=Sample point 3	10	4	0,44	44%
B=Sample point 4	16	7	0,58	58%
B=Sample point 5	11	4	0,42	42%
B=Sample point 6	2	1	0,20	20%
B=Sample point 7	6	3	0,43	43%

A= Sample point 1
a= 8

Dice Coefficient: $\mathbf{Cs=2j(a+b)^{-1}}$, were **j** is the number of bands common to samples **A** and **B**, and **a** and **b** are the total number of bands in sample **A** and **B**, respectively. This index ranges from 0 (no common bands) to 1 (100% similarity of band patterns).

Additional information gathered from D13.53 and D13.55:

Pathogen detection using culture-dependent methods: No target pathogens (*E.coli, P.aeruginosa, C.jejuni, enterococci, and Salmonella*) were found using culture dependent methods.

Pathogen detection using culture-independent methods: DNA from *C.jejuni* (point 6), enterococci (Point 6) and *P.aeruginosa* (Point 5) were found in some water samples, but the signals obtained were too close to the detection limits of the assays or the amplification curves were irregular compared to positive controls.

Only one sampling point (Point 6. Hygienic sluice) was found to exhibit a similarity value below threshold value of 40%, which is discussed as a possible critical control point.

Actually we are still waiting on the answered questionnaire from partner 33 for the complete evaluation of this SME. It was not possible to arrange another sampling period with this SME. Nevertheless, we would recommend changing hoses in higher frequency.

SPANISH SMEs RESULTS

Water samples were taken at the two Spanish SMEs and filtered in the laboratory of the Universidad de Burgos (Partner17). The samples were transported frozen to Germany. Only molecular biology culture independent

methods were applied for the evaluation of these SMEs. Therefore, a direct correlation between positive cultivation result and the presence of bacterial DNA targets from living bacteria, viable but not cultivable bacteria or from dead bacteria could not be performed.

Firstly, the PCR efficiencies were significantly reduced indicating the presence of inhibitors. The discussion with the Spanish partners and the evaluation of the answered questionnaire gave hints for the presence of natural inhibitors derived from the raw water sources. It is commonly known that water obtained from marshes has a high quantity in humic acids inhibiting PCR analyses. In the case of both Spanish food industries samples PCR inhibitors were found and PVPP was used to remove them.

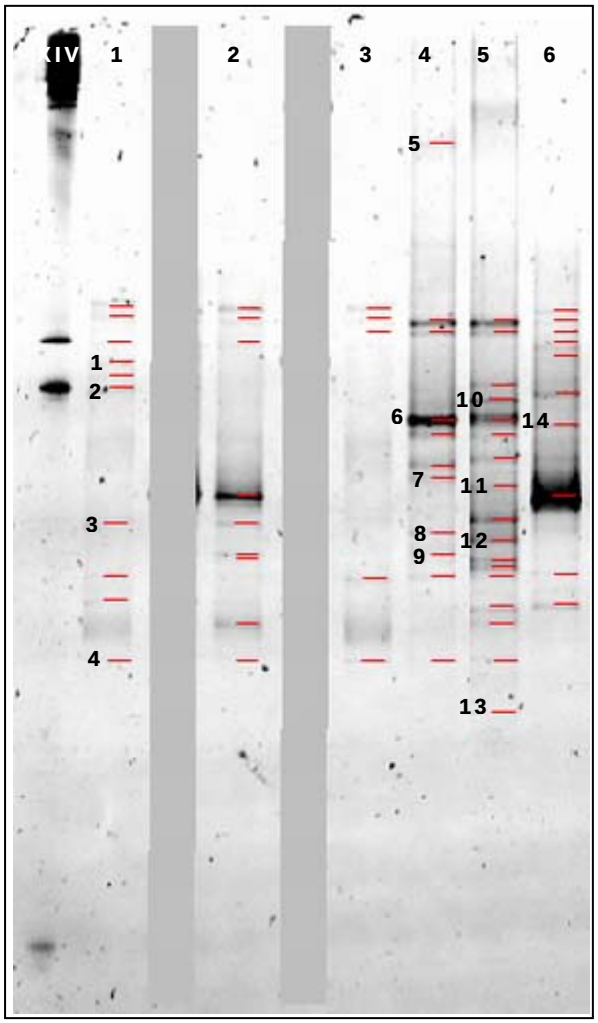
4.2.3 Spanish poultry industry

Water samples were taken at six different points from the industry where water is used in direct or indirect food processing.

1. Entry deposit of state water
2. Hand washbasin of cutting room
3. Hygienic sluice of sausages elaboration room
4. Shower after feather removal
5. Neck shower
6. Hand washbasin of elaboration room

The following DGGE gel picture shows the DNA fingerprints of the different sampling points at the Spanish poultry SME. Representative DNA bands were excised and sequenced for bacteria identification (see numbers in the gel). The sequencing results from database alignments are listed in the table beside.

Primers used for PCR-DGGE: **341F-907R**



1	Rickettsiales	α-proteobacteria
2	Sphingomonas	ß-proteobacteria
3	Bacillus sp	Firmicutes
4	Propionibacterium	Actinobacteria
5	Uncultured bacteria	γ-proteobacteria
6	Legionella spp.	γ-proteobacteria
7	Janthinobacterium	
8	Stenotrophomonas sp	γ-proteobacteria
9	Stenotrophomonas sp	γ-proteobacteria
10	Acinetobacter	γ-proteobacteria
11	Sphingomonas	α-proteobacteria
12	Xanthomonas	γ-proteobacteria
13	Rhodococcus	
14	Uncultured bacteria	α-proteobacteria

	Sampling point of poultry industry	Total DNA bands	Sequenced bands	Bacterial classes (number of sequenced bands)		
				α-Proteobacteria	*ß-Proteobacteria*	*γ - Proteobacteria*
WATER	1. Entry deposit of state water	10	4	1	1	-
	2. Hand washbasin of cutting room	9	3	-	-	1
	3. Hygienic sluice of sausages elaboration room (warm water)	5	1	1	-	-
	4. Shower after feather removal	11	6	6	-	-
	5. Neck shower	17	9	1	1	4
	6. Hand washbasin of elaboration room (warm water)	10	1	1	-	-
	Total sequenced bands		**14**			

Most of the sequenced DNA belongs to the *α-Proteobacteria* and *γ-Proteobacteria* subclasses.

Some opportunistic bacteria were aligned as: *Sphingomonas*, *Bacillus sp* (only three *Bacillus* species are clinically relevant, but these were not identified), *Acinetobacter sp* and non-pneumophila *Legionella*.

	b	**j**	Dice coefficient (**Cs**)	Similarity between A and B
A=B=Sample point 1	10	10	1,00	100%
B=Sample point 2	9	5	0,53	53%
B=Sample point 3	5	4	0,53	53%
B=Sample point 4	11	3	0,29	29%
B=Sample point 5	17	6	0,44	44%
B=Sample point 6	10	4	0,50	50%

A= Sample point 1
a= 10

Dice Coefficient: $\mathbf{Cs=2j(a+b)^{-1}}$, were **j** is the number of bands common to samples **A** and **B**, and **a** and **b** are the total number of bands in sample **A** and **B**, respectively. This index ranges from 0 (no common bands) to 1 (100% similarity of band patterns).

Additional information gathered from D13.55:

Pathogen detection using culture-independent methods: DNA from *E.coli* (point 4) and *P .aeruginosa* (Point 3,4 and 6) were found in some water samples, but the signals obtained were close to the detection limits of the assays.

Only one sampling point (Point 4. Shower after feather removal) was found to exhibit a similarity value below threshold value of 40%, which is discussed as a possible critical control point.

A correlation between the molecular biology detection of pathogens and extended populations shifts during PCR-DGGE was not observed. Although some positive pathogenic bacteria results were seen after DNA specific amplification, these bacteria were not identified in the DNA fingerprints. We cannot say if the detected DNA comes from live or dead cells. And in the case of this industry we do not have the back up of the conventional plating techniques. Nevertheless with the obtained results we would recommend changing hoses in higher frequency and give special attention in a correct rinse and disinfection of the machine at point 4.

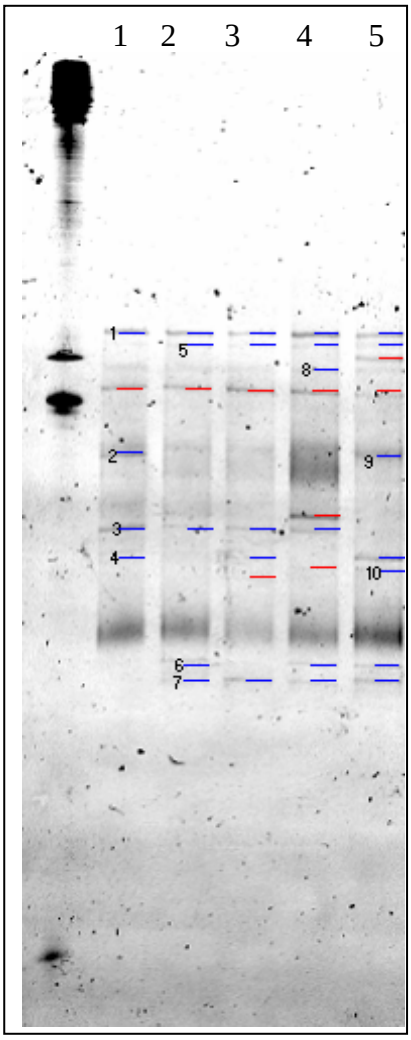
4.2.4 Spanish dry cured ham industry

Water samples were taken at five different points from the industry where water is used in direct or indirect food processing.

1. Hygienic sluice
2. Salt wash off
3. Entry of state water
4. Hand washbasin of deboneing room
5. Hand washbasin of packaging room

The following DGGE gel picture shows the DNA fingerprints of the different sampling points at the Spanish dry-cured ham SME. Representative DNA bands were excised and sequenced for bacteria identification (see numbers in the gel). The sequencing results from database alignments are listed in the table beside.

Primers used for PCR-DGGE: **341F-907R**



1	Uncultured bacteria	γ-proteobacteria
2	Uncultured bacteria	ß-proteobacteria
3	Bacillus sp	Firmicutes
4	Stenotrophomonas sp	γ-proteobacteria
5	Pseudomonas sp	γ-proteobacteria
6	Propionibacterium	Actinobacteria
7	Enterobacter	γ-proteobacteria
8	Uncultured bacteria	γ-proteobacteria
9	Pseudomonas sp	γ-proteobacteria
10	Stenotrophomonas sp	γ-proteobacteria

	Sampling point of ham industry	Total DNA bands	Sequenced bands	Bacterial classes (number of sequenced bands)	
				ß-Proteobacteria	*γ -Proteobacteria*
WATER	**1. Entry of state water**	7	5	**-**	**4**
	2.Hygienic sluice (warm water)	5	4	**1**	**2**
	3. Salt wash off	6	5	**-**	**3**
	4. Hand washbasin of deboneing room	9	6	**-**	**4**
	5. Hand washbasin of packaging room	9	7	**-**	**6**
	Total sequenced bands		**10**		

Most of the sequenced DNA belongs to the γ-Proteobacteria subclass.

Some opportunistic bacteria were aligned as: Bacillus sp, and Enterobacter .

	b	**j**	Dice coefficient (**Cs**)	Similarity between A and B
A=B=Sample point 1	7	7	1,00	100%
B=Sample point 2	5	4	0,67	67%
B=Sample point 3	6	5	0,77	77%
B=Sample point 4	9	5	0,63	63%
B=Sample point 5	9	5	0,63	63%

A= Sample point 1
a= 7

Dice Coefficient: $\mathbf{Cs=2j(a+b)^{-1}}$, were **j** is the number of bands common to samples **A** and **B**, and **a** and **b** are the total number of bands in sample **A** and **B**, respectively. This index ranges from 0 (no common bands) to 1 (100% similarity of band patterns).

Additional information gathered from D13.55:

Pathogen detection using culture-independent methods: DNA from *P.aeruginosa* (Point 2, 3 and 5) were found in some water samples, but the signals obtained were close to the detection limits of the assays.

When the bacterial population of the water samples were compared with the entry water, no sampling point was found to be a possible critical control point.

A correlation between the molecular biology detection of pathogens and extended populations shifts during PCR-DGGE was observed.

Although some positive pathogenic bacteria results were seen after the use of culture independent methods, again we cannot say if the detected DNA comes from live or dead cells. In the case of this industry we do not have the back up of the conventional plating techniques. Nevertheless, with the obtained results we would recommend changing hoses in higher frequency and due to the fact that the drinking water pipelines are 20 years old a disinfection of them would be also recommended.

Questionnaire for SMEs Concerning Drinking Water Distribution

Partner 31 – German Dairy SME

Sampling Points:

1. Income of state water
2. Lactic acid tank
3. Portioner
4. Hand washbasin
5. Maturation room
6. Feta packaging

Origin and Processing of Drinking Water

1. Which waterworks supply you with drinking water? Water association and water supplier Haslach, Neukirch, Germany.
2. Which types of raw water are used by the waterworks and how is the raw water processed? Exclusively ground water without treatment.
3. Which disinfection measures are taken in drinking water processing? None.

Drinking Water Distribution

4. Does your company carry out a secondary treatment of the drinking water (e.g. additional disinfection, softening, etc.)? No
5. Which materials were used for the drinking water pipelines and how old are the pipelines and connections? Exclusively stainless steel.
6. Did you use several materials and in which order? Stainless steel.
7. Did you renew your drinking water pipelines while using the building? Not in the last years
8. Did you detect any damage of the water pipelines in the last years (e.g. pipe ruptures, corrosion, etc.)? No

9. Is the microbiological control of your drinking water carried out internally or externally? Externally and internally At which intervals? Both are taken every 6 months.
10. Do you have a current version of a plan of all drinking water pipelines and flow directions of the drinking water in your production buildings? No
11. What do you think is critical to drinking water hygiene? We think that there is no problem with the water that we use. The water that we employ has not ever been the cause of a hygienic problem, and wholly satisfies the German Drinking Water Ordinance requirements.

Additional Aspects of Drinking Water Distribution

12. Do you use hoses or additional supplements for the cleaning and/or processing of foods? Yes on sampling point 3. Which material do they have? PVC
13. Are you able to provide information on your water consumption and allocate the amounts of water consumed to the production lines? Income of state water: ca. 150m³/Tag
14. Do you also use warm water in production? Yes at points 2 and 4.
15. How is the warm water prepared and fed into the production line? By a plate-type heat exchanger. There is also a local additional mixing faucet for the generation of hot water at point 2
16. Do you spray or atomize water during production? Yes, the water sampled in point 5 is evaporated for the humidification of the air of the maturation room.
17. Is an emulsion prepared during production? No

Partner 29 – Spanish poultry SME

Water sampling points:

1. Income of state water (cold)
2. Hand washbasin of cutting room (cold)
3. Hygienic sluice of sausages elaboration room (warm)
4. Shower after feather removal (cold)
5. Neck shower (cold)
6. Hand washbasin of elaboration room (warm).

Origin and Processing of Drinking Water

1. Which waterworks supply you with drinking water? Municipal water provision of Burgos

2. Which types of raw water are used by the waterworks and how is the raw water processed? Swamp
3. Which disinfection measures are taken in drinking water processing? Chlorine and ozonized water.

Drinking Water Distribution

4. Does your company carry out a secondary treatment of the drinking water (e.g. additional disinfection, softening, etc.)? No
5. Which materials were used for the drinking water pipelines and how old are the pipelines and connections? Stainless steel and PVC, they are 4 years old.
6. Did you use several materials and in which order? Stainless steel and PVC.
7. Did you renew your drinking water pipelines while using the building? No
8. Did you detect any damage of the water pipelines in the last years (e.g. pipe ruptures, corrosion, etc.)? No
9. Is the microbiological control of your drinking water carried out internally or externally? Externally At which intervals? Annual
10. Do you have a current version of a plan of all drinking water pipelines and flow directions of the drinking water in your production buildings? No
11. What do you think is critical to drinking water hygiene? The dirtiness of pipes and faucets

Additional Aspects of Drinking Water Distribution

12. Do you use hoses or additional supplements for the cleaning and/or processing of foods? Yes. Which material do they have? PVC
13. Are you able to provide information on your water consumption and allocate the amounts of water consumed to the production lines?

Factory: 12 m^3 monthly

Incubator: 175 m^3 monthly

Global slaughter house: 6.034 m^3 monthly

14. Do you also use warm water in production? Yes, for the cleaning
15. How is the warm water prepared and fed into the production line? Gas boiler
16. Do you spray or atomize water during production? Yes
17. Is an emulsion prepared during production? No

Partner 34 – Spanish dry-cured ham SME

Water sampling points:

1. Entry deposit of state water
2. Hand washbasin of cutting room
3. Hygienic sluice of sausages elaboration room
4. Shower after feather removal
5. Neck shower
6. Hand washbasin of elaboration room

Origin and Processing of Drinking Water

1. Which waterworks supply you with drinking water? Aqualia S.A.
2. Which types of raw water are used by the waterworks and how is the raw water processed? Groundwater. It takes a chlorination treatment.
3. Which disinfection measures are taken in drinking water processing? Chlorination

Drinking Water Distribution

4. Does your company carry out a secondary treatment of the drinking water (e.g. additional disinfection, softening, etc.)? No
5. Which materials were used for the drinking water pipelines and how old are the pipelines and connections? The pipelines and connexions are 20 years old
6. Did you use several materials and in which order?
7. Did you renew your drinking water pipelines while using the building? No
8. Did you detect any damage of the water pipelines in the last years (e.g. pipe ruptures, corrosion, etc.)? No
9. Is the microbiological control of your drinking water carried out internally or externally? Externally. At which intervals? microbiological controls are done every four month period.
10. Do you have a current version of a plan of all drinking water pipelines and flow directions of the drinking water in your production buildings? No
11. What do you think is critical to drinking water hygiene? The treatment done by the water supplier and the conditions of the pipelines.

Additional Aspects of Drinking Water Distribution

12. Which hose materials are possibly connected to the water pipelines and used for cleaning or food processing? Yes, occasionally rubber hoses are used for cleaning.
13. Are you able to provide information on your water consumption and allocate the amounts of water consumed to the production lines?

14. Do you also use warm water in production? No (NOTE: as we took the samples we noticed that hand wash basins have warm and cold water)
15. How is the warm water prepared and fed into the production line? We do not use warm water in the production
16. Do you spray or atomize water during production? No (NOTE: there is a step of the production process where they spray water to wash the salt out of the ham)
17. Is an emulsion prepared during production? No

4.3 General conclusions

After the analytical evaluation of the water samples taken at each SME, some possible water-derived critical control points were found. After the evaluation (i.e. bacterial population analysis, specific pathogen detection, DNA sequencing, questionnaire) although no real critical control point was found, some hygienic recommendations were done.

It is concluded that a good hygienic practice for water supply and usage are done at these SMEs. In case of the two German SMEs, our extended investigations and the regular hygienic surveillance of the waterworks as well as food industries by the German hygiene authorities demonstrated that they meet the high German drinking water standards. Inconsequence, the two SMEs may serve as a model for good hygienic practice in food industry.

It should be considered that the culture independent techniques used here cannot distinguish among viable, viable but not cultivable, injured, and dead cells.

Nevertheless, the use of these culture-independent techniques has a high applicability for the identification of bio-hazards and critical control points at all stages of food production where water is involved.

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