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One HEALTH EJP
TOXOSOURCES

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Aula Marotta
Istituto Superiore di Sanità

A European multicenter survey on *Toxoplasma gondii* oocysts contamination in RTE salads: a true risk for consumers?

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Toxoplasma gondii: One Health paradigm

Causes significant diseases in animals and humans

Infective stage and trasmission:

Horizontal

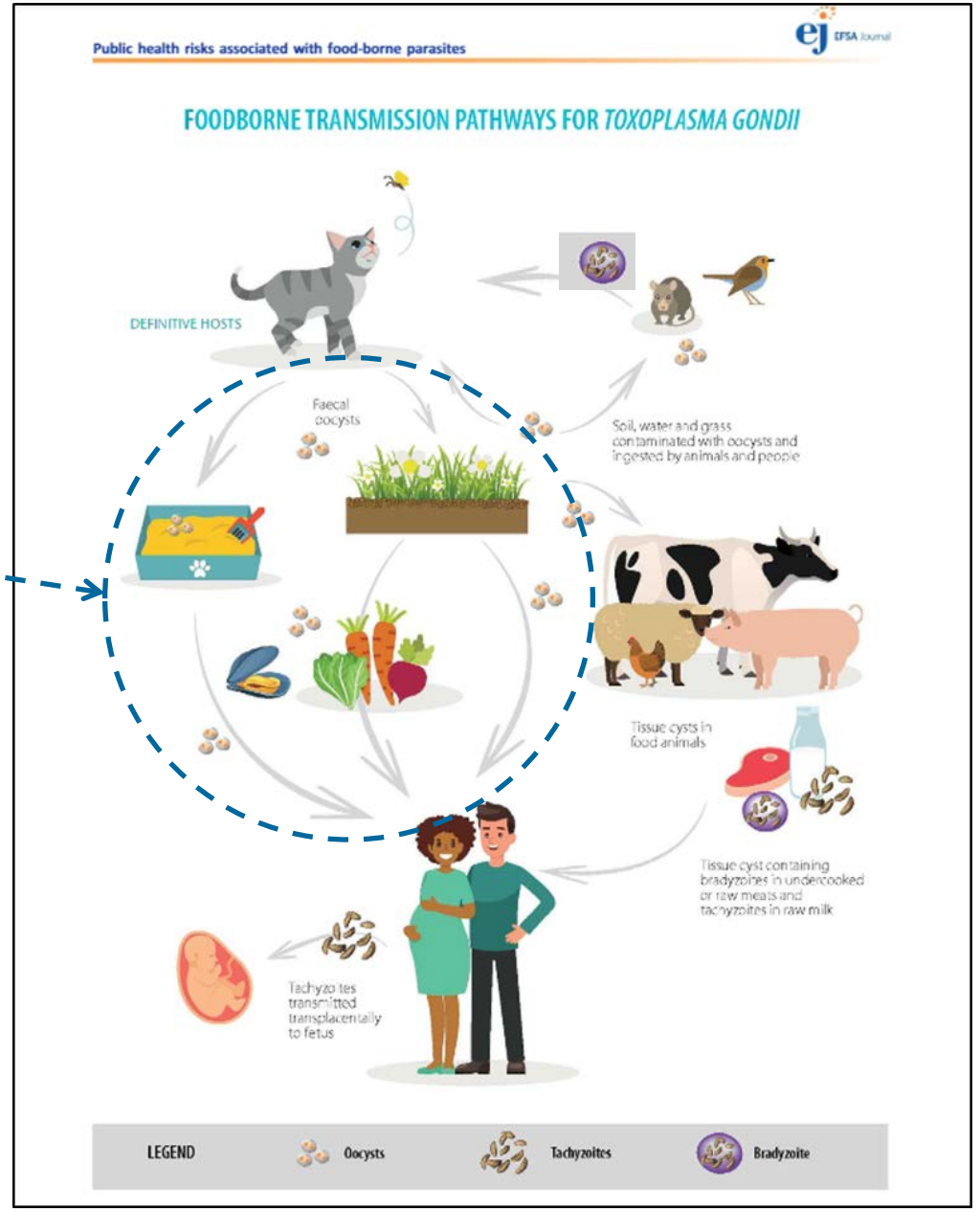
- **Tissue cysts** → raw or undercooked meat
- **Oocysts** → soil, water, vegetables, mussels
- **Tachyzoites** → raw milk

Vertical

- **Tachyzoites** → transplacental

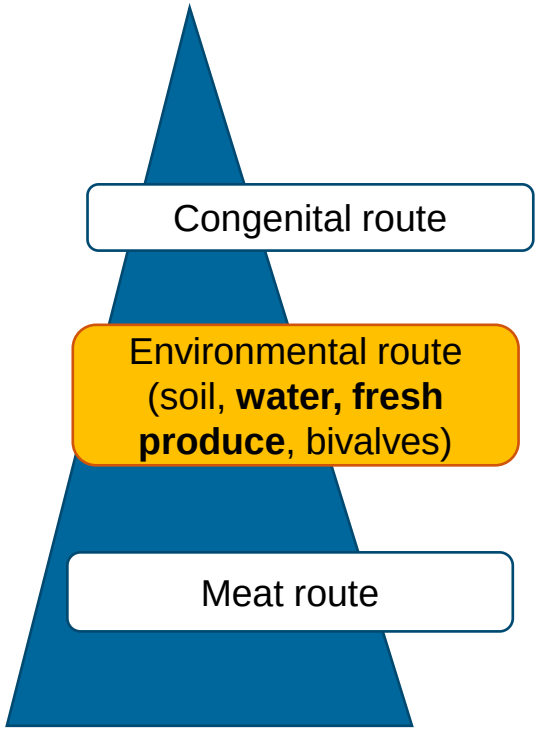
Globally 40–60% of acquired *T. gondii* infections are foodborne

3nd most important foodborne parasite worldwide and 2nd in Europe



Source: EFSA Journal 2018;16(12):5495

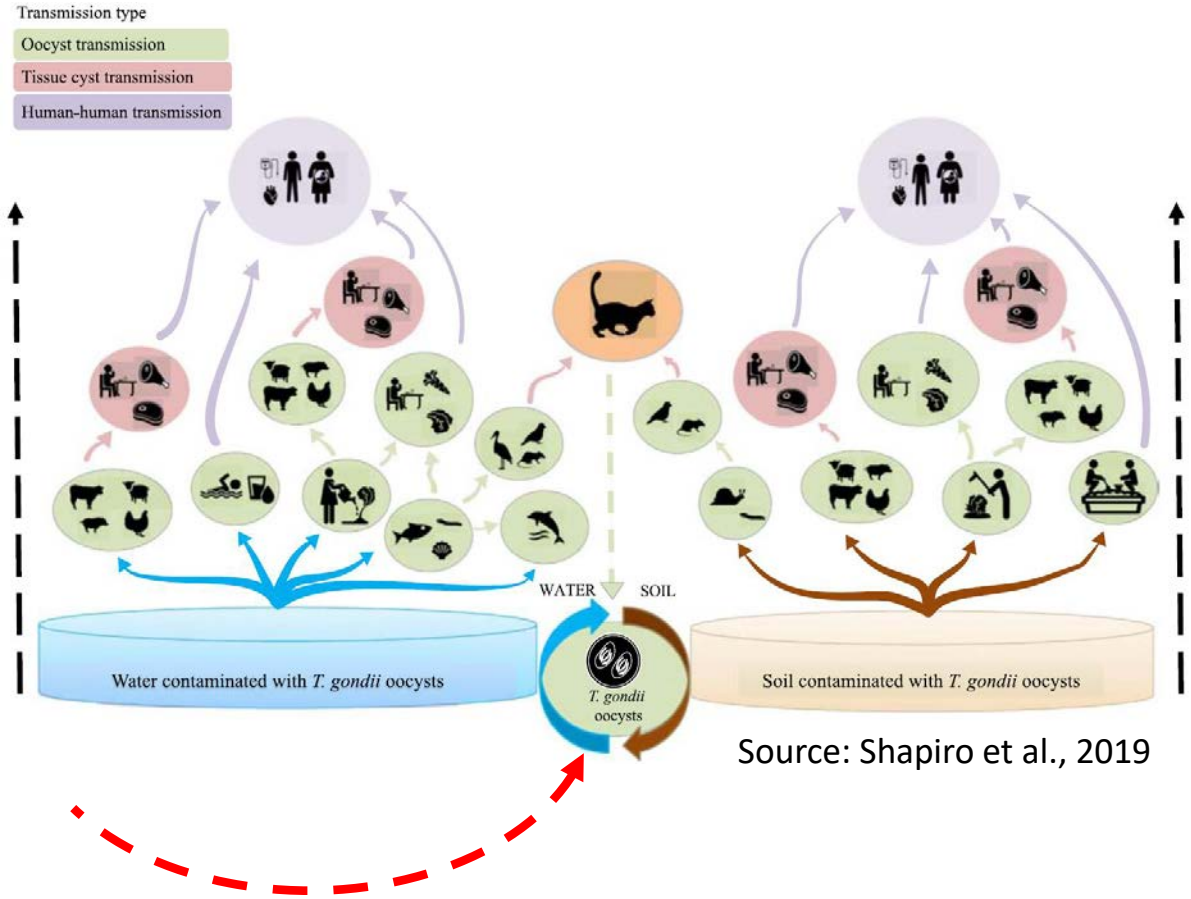
Transmission routes



133 congenitally reported cases in EU/ EEA in 2020

2nd food vehicle
44.1% of worldwide outbreaks are oocyst-related

1st food vehicle
Small ruminants, pork, beef…



Source: Shapiro et al., 2019

Environmental contamination with *T. gondii* oocysts is understudied

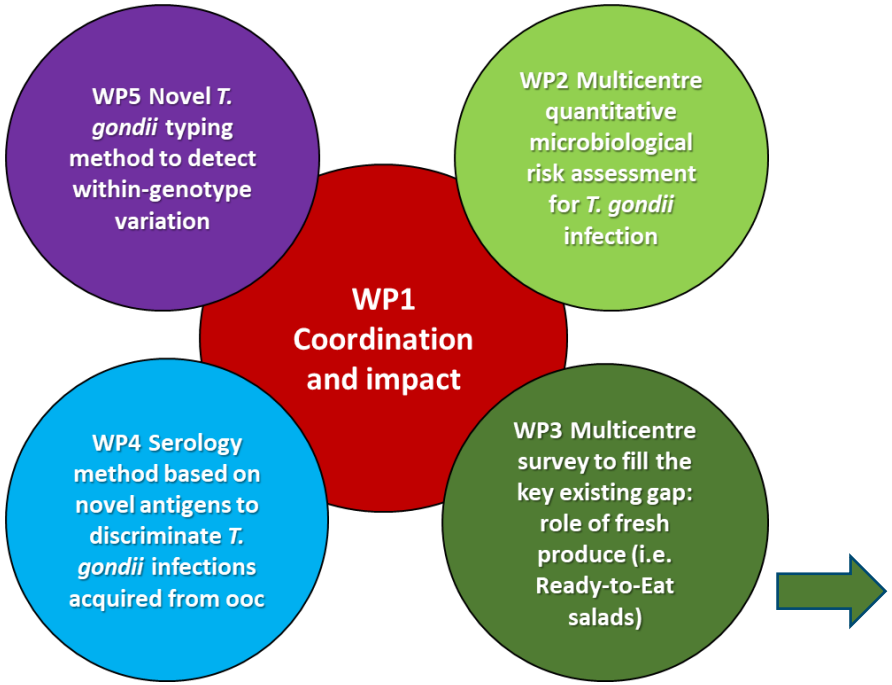
Limited information on the contribution of the different environmental sources: e.g. testing for parasite contamination in fresh produce is neither regulated nor mandatory

Lack of standardized/fully validated detection methods: development of effective intervention strategies hampered

TOXOSOURCES *Toxoplasma gondii* sources quantified

One Health EJP Joint Research Project
21 One Health EJP partners and several external partners
Project Leader: Pikka Jokelainen. SSI. Denmark
Project Co-Leader: Joke van der Giessen. RIVM. The Netherlands

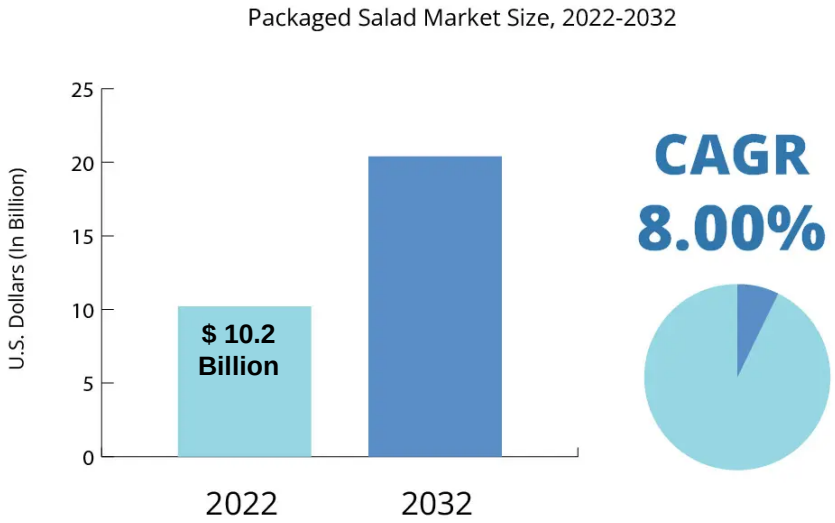
What are the relative contributions of the different sources of *Toxoplasma gondii* infection?

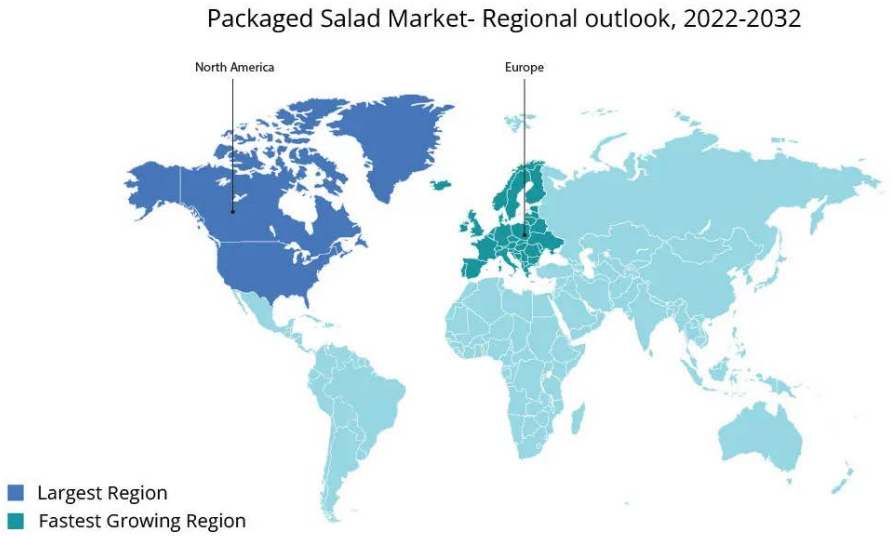


TOXOSOURCES WP3 aims to fill the knowledge gap concerning the relevance of **fresh produce** contaminated with T. gondii oocysts as an infection source for humans

- **To provide an overview of *T. gondii* oocysts in fresh produce and the environment (with a focus on Europe)**
- **To identify and validate the most appropriate procedure to detect *T. gondii* oocysts in fresh produce**
- **To conduct a multicenter pilot survey on *T. gondii* oocysts contamination in RTE salads in Europe**

Ready-to-eat (RTE) foods such as fresh vegetables, fruit, herbs and sprouts are increasingly consumed in industrialized countries, are perceived as healthy, easy to consume and safe!

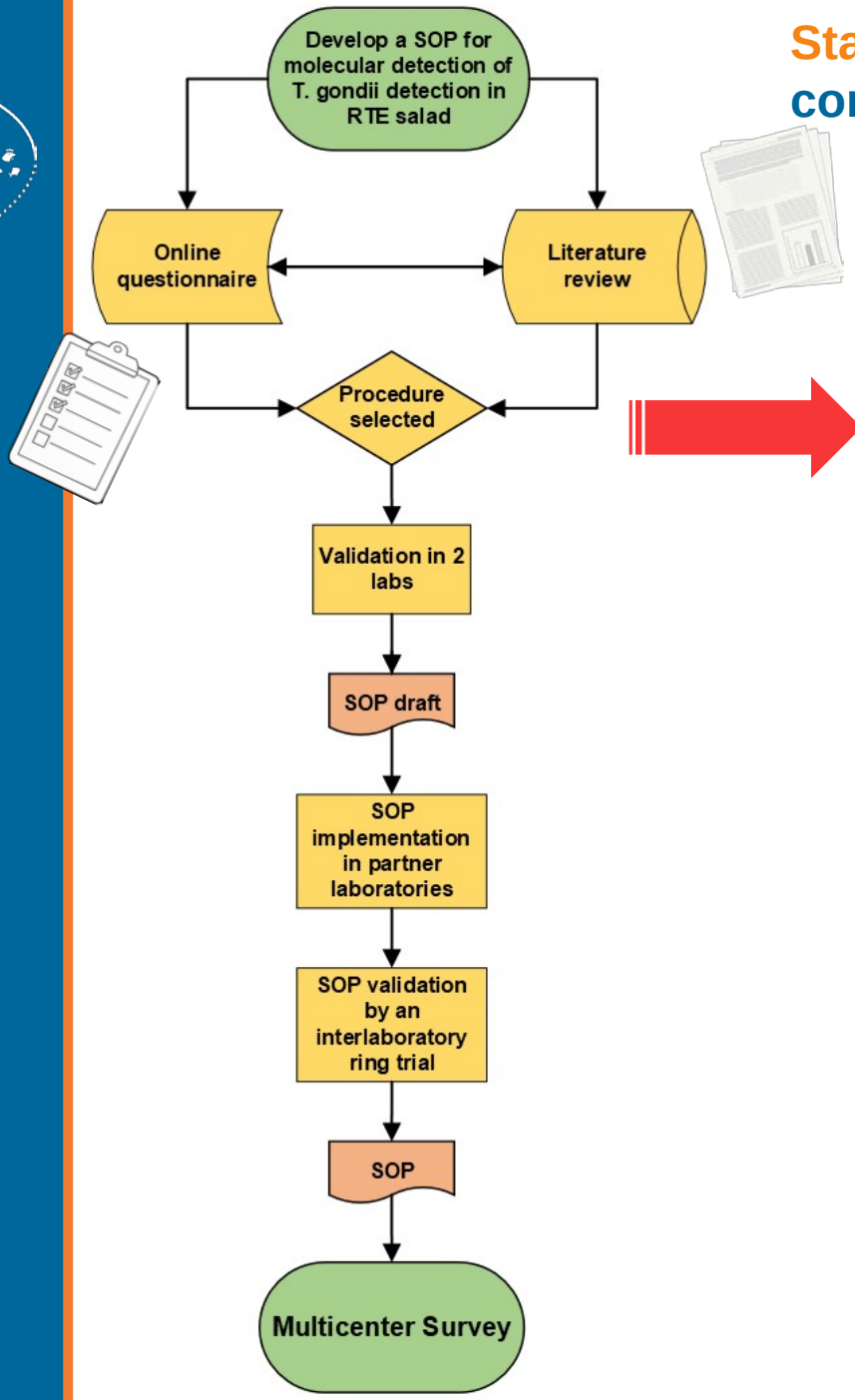




Credits: https://www.reportsanddata.com/report-detail/packaged-salad-market

However they can be contaminated by pathogenic parasites at any stage of processing and have been associated to parasite foodborne outbreaks worldwide.

Standard operating procedure (SOP) to ensure reliable and comparable data in a Multicenter Survey



DNA amplification assay: Probe-based TIPLEX qPCR targeting the 529RE (high sensitivity) and the B1 (high specificity) + IAC (synthetic gene)

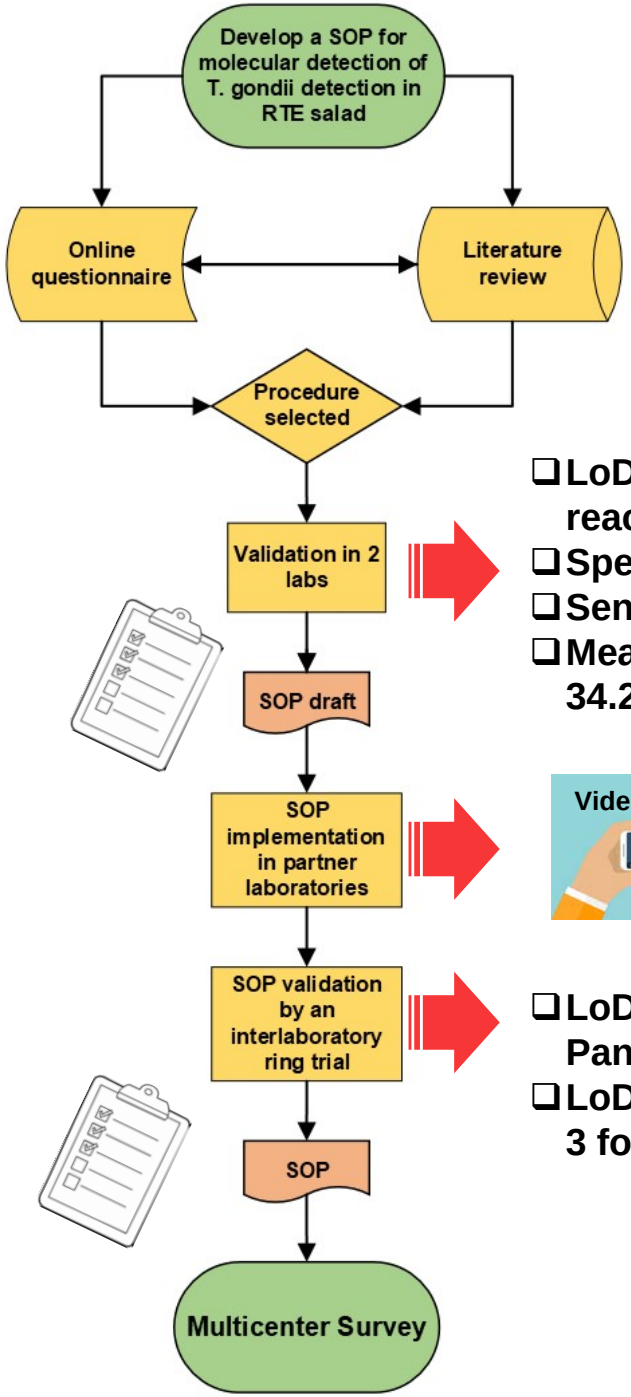
Type of sample: RTE Leafy green mixed salad

Amount of sample to be tested: 30 g

Oocyst recovery: Washing by stomaching in filter-bags with glycine 1M pH 5.5 and pelleting by centrifugation (2500 x g 10 min)

SOP

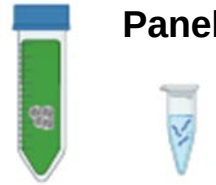
DNA extraction: Beat-beating based commercial kit for soil vs freeze and thaw in combination with silica spin columns kits



- LoD = 10 *T. gondii* oocysts/30g matrix (1 oocyst equivalent/qPCR reaction).
- Specificity 100%
- Sensitivity (% samples positives) = 100% for 529RE; >70% for B1
- Mean Cq values (10 oocysts spiking) = 31.31±3.35 for 529RE; 34.29±3.68 for B1.

- LoD = 10 *T. gondii* oocysts/30 g matrix by >80% of labs with Panels 1 for 529RE
- LoD = 10 *T. gondii* oocysts by 100% of labs with Panels 2 and 3 for 529RE

Panel 1



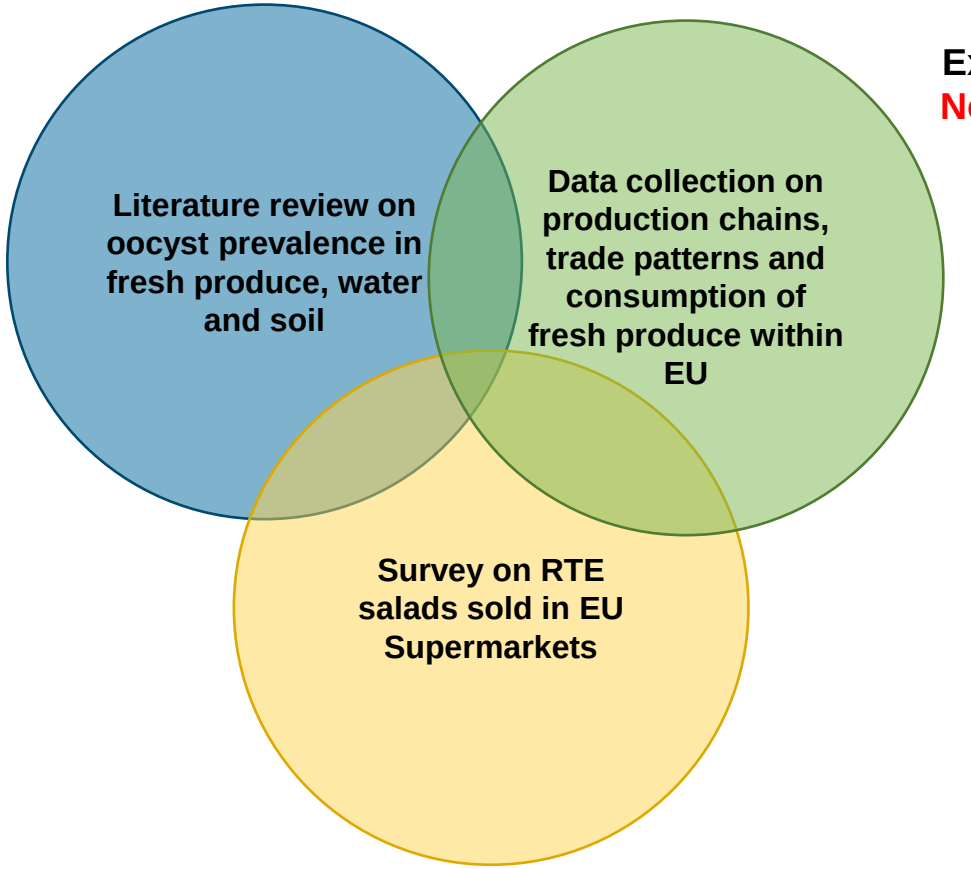
Panel 3

Panel 2

Design of a risk-based sampling strategy

- ❑ To define the most representative RTE salad
- ❑ To define the sample size

Detection rates <10%
(range 0.3%-50%)



Request for Information Exchange to EFSA focal point
No useful data to be extracted

A survey on producers at National level by online questionnaire:
Answers from very few companies

Data were collected from 10 countries:
Poland, UK, France, Spain, Germany, Norway, Czechia, Portugal, Italy, Denmark
Useful information!

Sampling plan

Sample types:

- Baby leaves -mixes (**BABY LEAVES**): Spinach baby, arugula, baby lamb's lettuce; baby red and green lettuce
- Cut salads -mixes (**CUT SALADS**): Frisée (curly escarole); Radicchio (red); Endive; any green lettuce (e.g. Iceberg, roman, etc.).

Mixes should containing 3 to 4 type of the listed vegetables.
At least 2 different brands of each type of salad mix to be selected

Where to sample

- Supermarkets
- Stores

Timing of sampling

- Sample collection done regularly throughout each season
- Weekly sampling

Number of samples

- 40 baby leaves + 40 cut salad mixes / country / season
- 320 samples/country

Power calculation

- ❑ considering a predicted prevalence of 1% (confidence level 95%) → seasonal prevalence of oocyst contamination of RTE salad (baby leaves and cut salad) to be estimated to a precision of 1%-2%
- ❑ the power to detect medium and large effect sizes >90% (95% significance)

Sample metadata collection on Excel sheet

Sample Number	Sampling Country	Brand	Product name	Category*	Salad Composition	Organic (Y/N)	Country Origin	Producer according to package	Barcode	Packaging facility (e.g. by barcode)	Packaging Country (e.g. by barcode)	Date of packaging	LOT OR Batch OR Time stamp	replicates	Expiration date
IT-001	Italy	A	X	Cut salads	sugar loaf chicory, red round radicchio, curled endive, endive escarole	N	Italy	A	3-083680-527736	Address	Italy	Unknown	B2870131108	0	23.10.2021

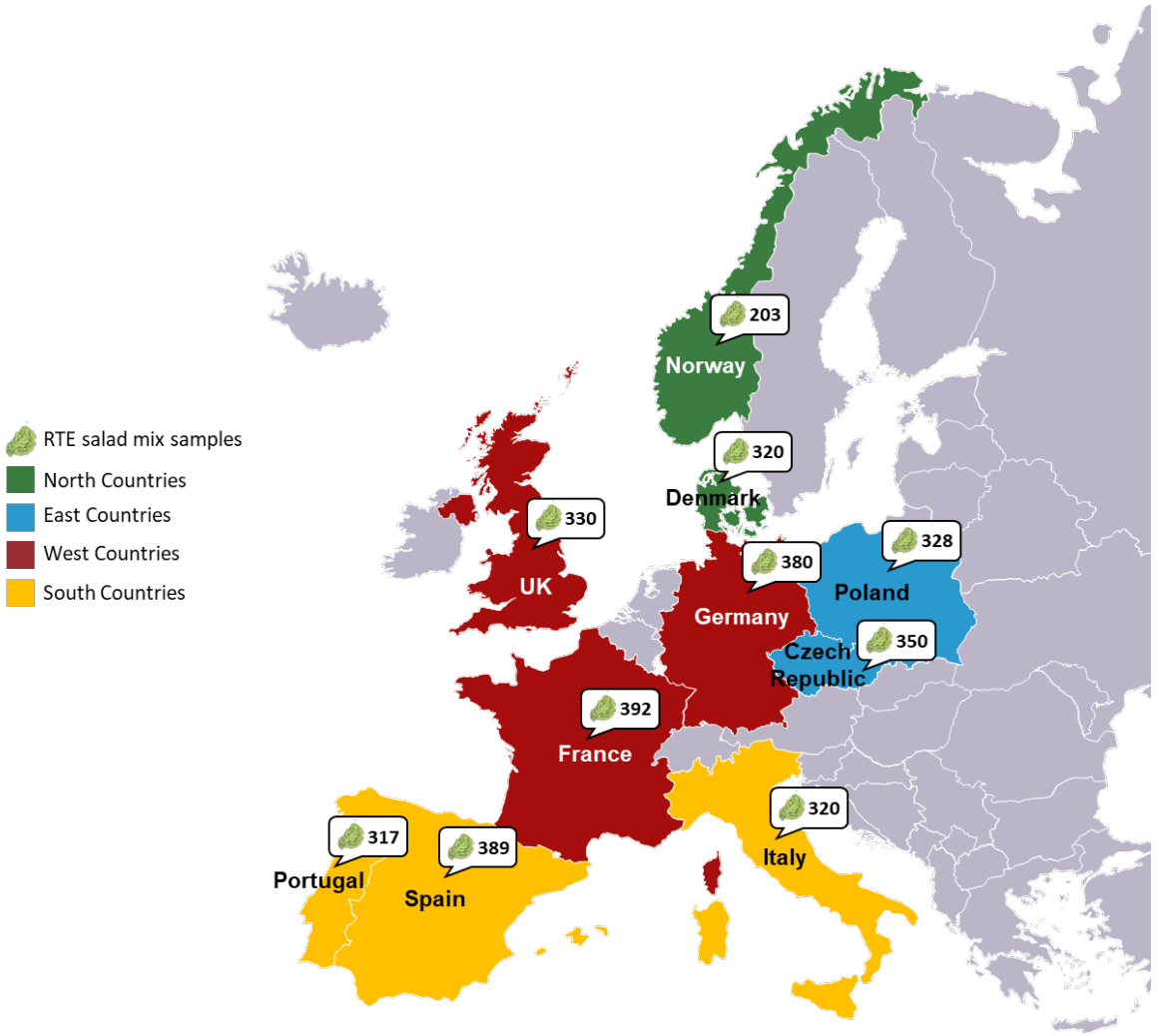
***Baby leaves OR Cut salads OR Other OR Mostly baby leaves OR Mostly cut salads**

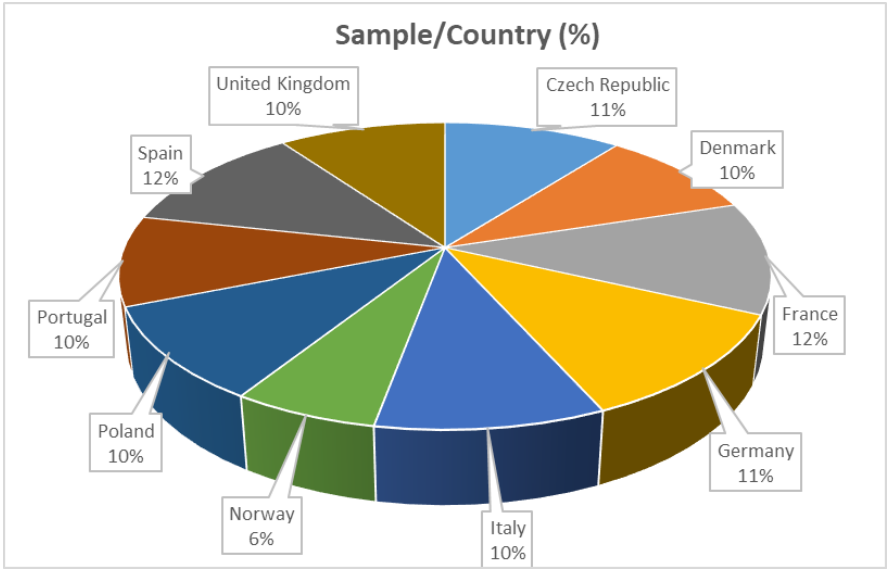
Date of sampling	Date of sample processing (washing)	Date of DNA extraction	DNA extraction Kit* (incl. Freeze-Thaw cycles)	DNA extraction Kit LOT number	Date of qPCR	qPCR Mastermix	qPCR Mastermix LOT number	DNA template dilution
16.10.2021	19.10.2021	12.11.2021	FastDNA™ SPIN Kit for Soil (MP Biomedicals)	138463 (exp. 31/10/2023)	16.11.2021	Roche, LightCycler Multiplex DNA Master no ROX 5X	4852800 (Exp. 31-03-2022)	undiluted

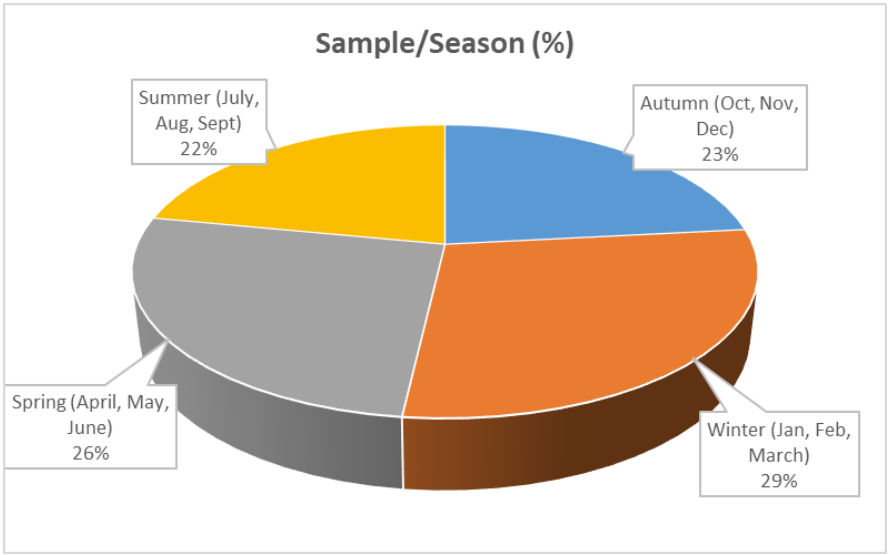
529-RE CTmean	529-RE CT-SD	529-RE Result	529-RE positive replicates of 3 replicates	B1 CTmean	B1 CT-SD	B1 Result	B1 positive replicates of 3 replicates	IAC CTmean	IAC CT-SD	IAC Result	IAC positive replicates of 3 replicates	Final Result	Comment	Standard curve (Efficency)	Standard curve (R2)	Standard curve Slope
0	0	negative	0	0	0	negative	0	23,07	0,21	positive	3	negative	Big pellets	B1=103.895%; 529RE=97.792%	NA	B1 =-3.232; 529RE=-3.376

- **12 months survey (Oct 2021-Sep 2022)**
- **10 European countries**
- **3329 samples processed**

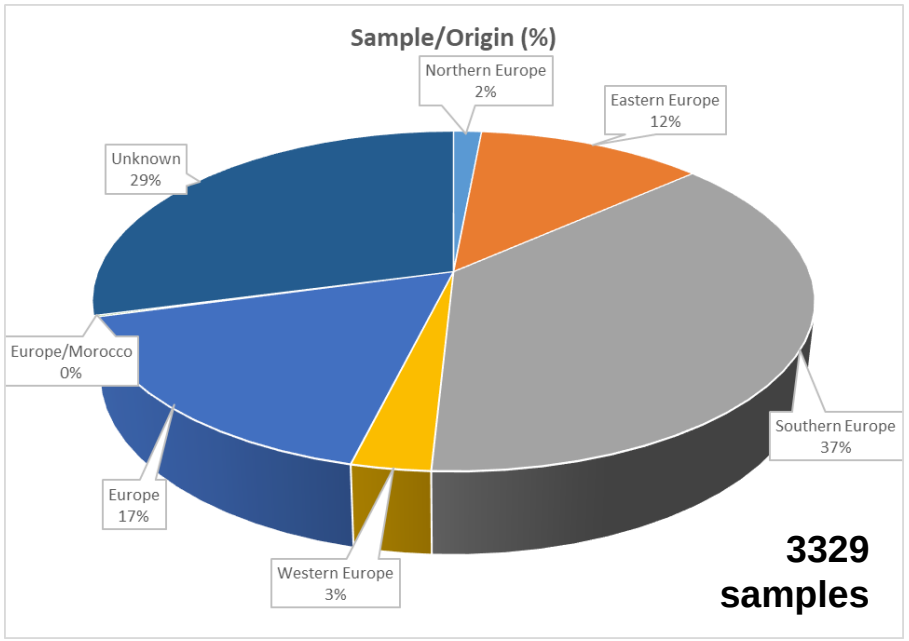
Baby leaves	1618
Cut salad	1691
Mixed	16
Unknown	4

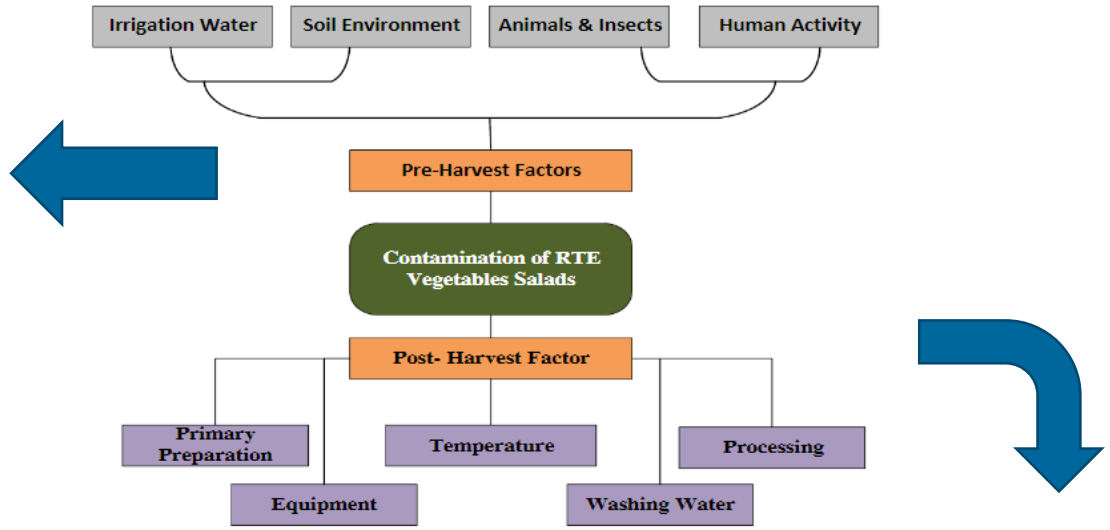






Sample origin and packaging facility

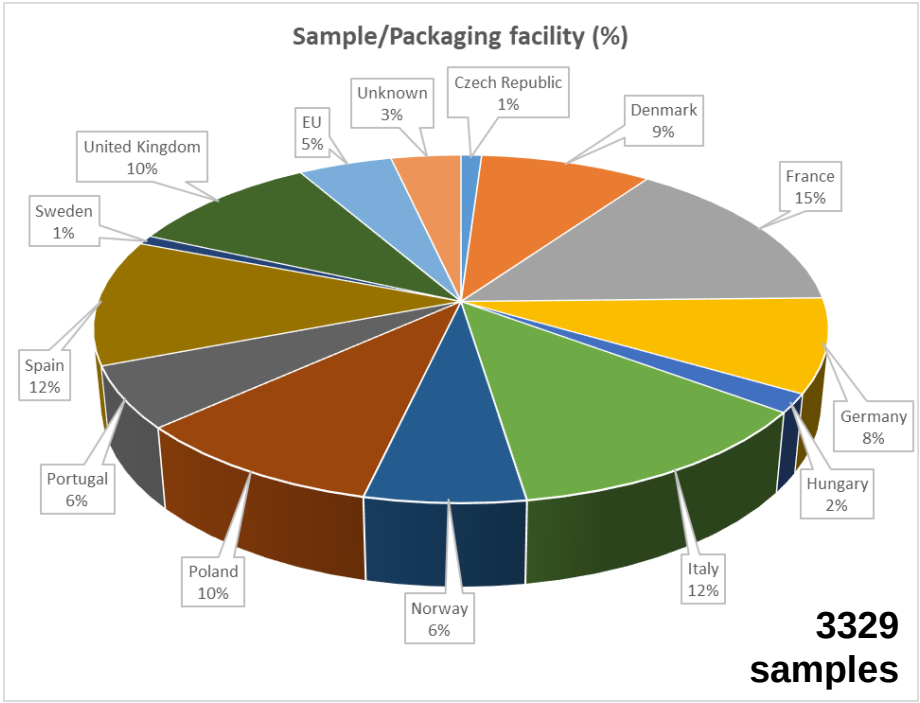




Surce:M., Dandago M. A., Igwe E. C., Salami K. D., DUJOPAS 7 (4a): 38-48, 2021

- 18.1% of EU total growing area is dedicated to the diverse group of leafy and stalked vegetables (such as lettuce, spinach, chicory, endives, asparagus, artichokes, etc.).
- ~50% of combined areas in Italy, Spain and Poland!.
- Export of fruit and fresh vegetables between Member States within the EU and to countries outside the EU was €36.8 billion vs €4.6 billion.

Source: Eurostat 2017

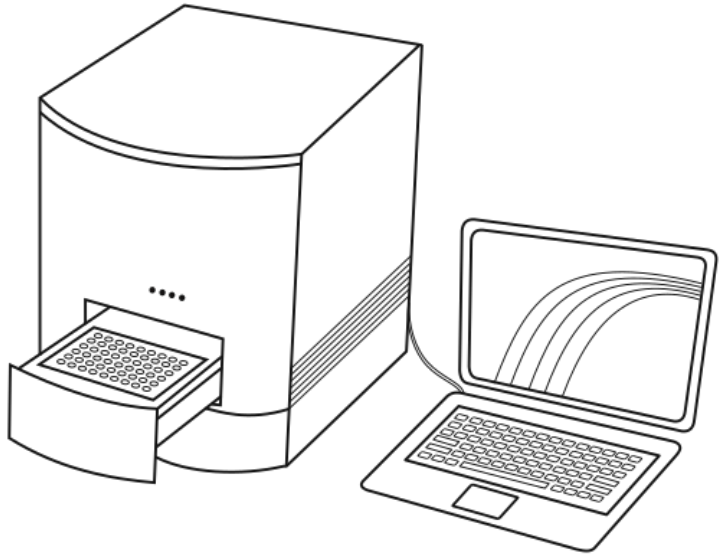


High variability in information reported on salad package among countries!

Triplex qPCR (B1+529RE+IAC) interpretation

POSITIVE

•amplification signal for at least one target (B1 or 529RE) for one or more sample replicates
•Ct ≤39 for at least one target (B1 or 529RE) for one or more sample replicates
•IAC reaction is positive. NOTE: In samples highly positive for *T. gondii* DNA, the Ct value for the IAC could be much higher, or even negative, compared to the positive and negative controls.



NEGATIVE

•no amplification signal for both targets (B1 and 529RE) in all sample replicates
•Ct >39 for both targets (B1 and 529RE)
•Ct values for IAC comparable (±1-2 Ct value) with the positive and negative controls.

NOT DETERMINED (e.g. inhibited reaction)

•no amplification signal for both targets (B1 and 529RE) in all sample replicates
•Ct >39 for both targets (B1 and 529RE)
•Ct >39 or generally much higher (Ct>3) compared to the positive and negative controls for the IAC

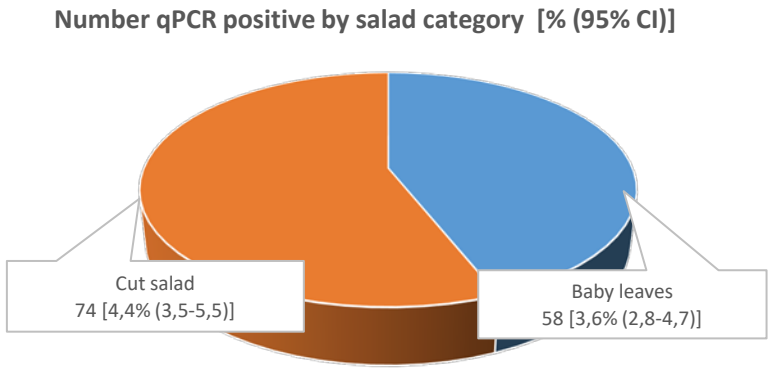
In this last event, dilute the DNA sample (e.g. 1:2-1:5) and repeat the test.

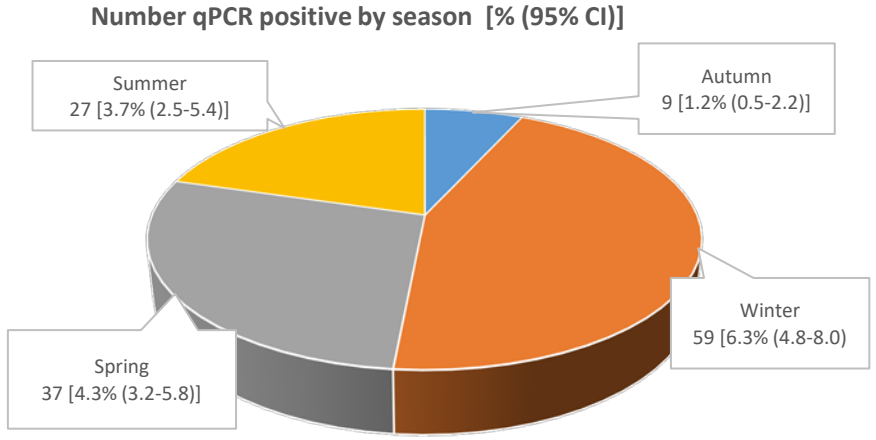
B1 (FAM chanel)	529RE (HEX chanel)	IAC (Cy5 chanel)	Result interpretation
positive	positive	positive	POSITIVE FOR T. GONDII
positive	negative	positive	POSITIVE FOR T. GONDII
negative	positive	positive	POSITIVE FOR T. GONDII
negative	negative	positive	NEGATIVE FOR T. GONDII
negative	negative	negative[a]	NOT DETERMINED

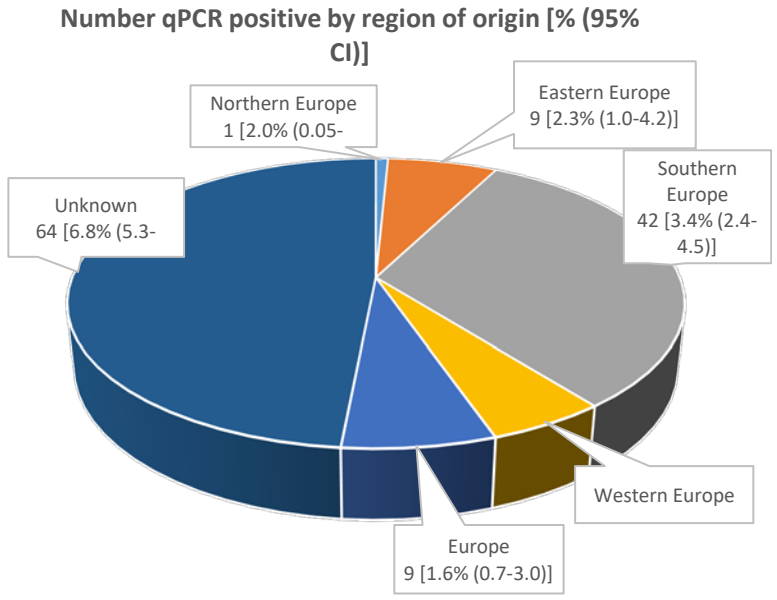
*Or if Ct values is much higher (Ct>3) compared to the positive and negative controls

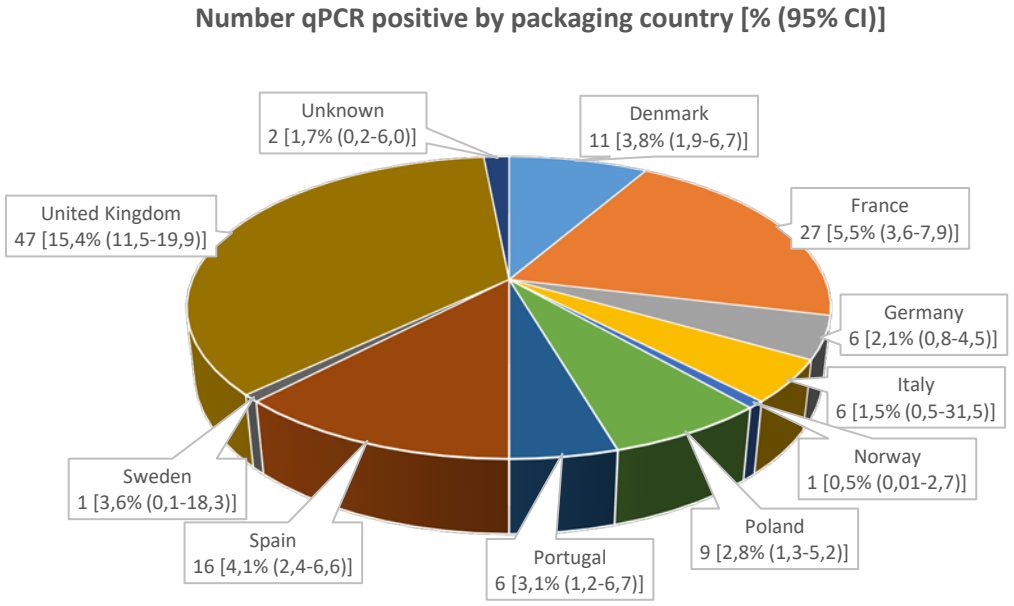
132 Toxoplasma positives samples based on qPCR
Prevalence = 4.0% (95% confidence interval: 3.4-4.7%; N=3295)

Country	Total number	Number qPCR positive	Percent qPCR positive (95% CI)
Czech Republic	350	0	0 (0-1.0)
Denmark	320	12	3.8 (2.0-6.5
France	392	24	6.1 (4.0-9.0)
Germany	376	9	2.4 (1.1-4.5)
Italy	317	6	1.9 (0.7-4.1)
Norway	202	1	0.5 (0.01-2.7)
Poland	327	9	2.8 (1.3-5.2)
Portugal	316	8	2.5 (1.1-4.9)
Spain	389	16	4.1 (2.4-6.6)
United Kingdom	306	47	15.4 (11.5-19.9)



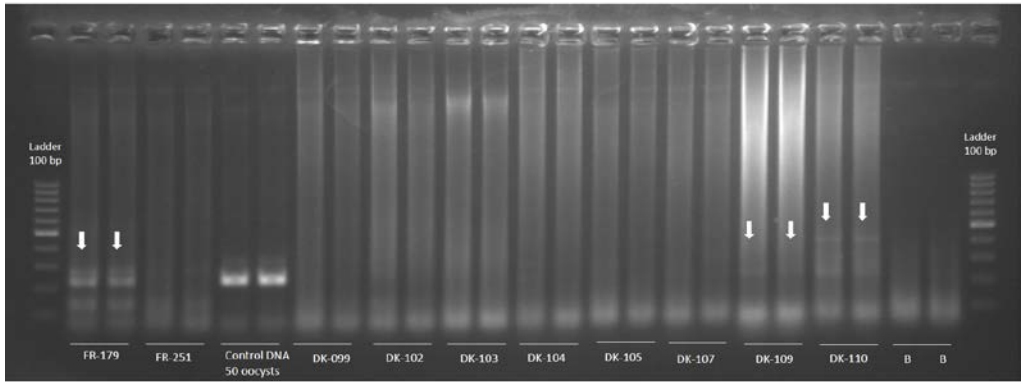


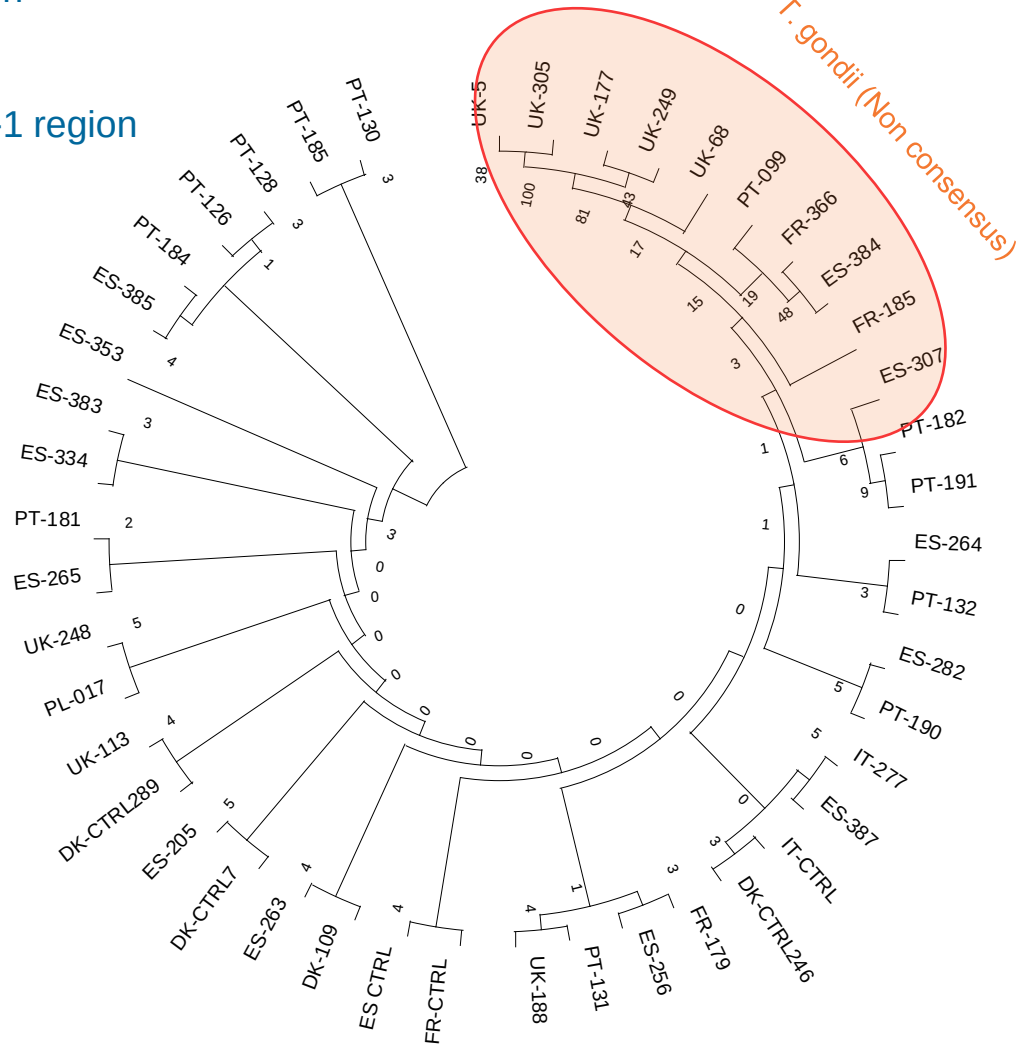




Independent confirmatory assays

- **129/132 (98%)** were additionally tested by single-tube nested PCR targeting the internal transcribed spacer 1 (ITS1) ribosomal region (Hurtado et al., 2001)
- **56/129 (43%)** were PCR amplified
- **36/129 (27%)** have PCR product which corresponded to the ITS-1 region of *T. gondii* upon sequencing = **1% Prevalence**





Bootstrap consensus tree inferred from 100 replicates using the Maximum Likelihood method and Tamura-Nei model

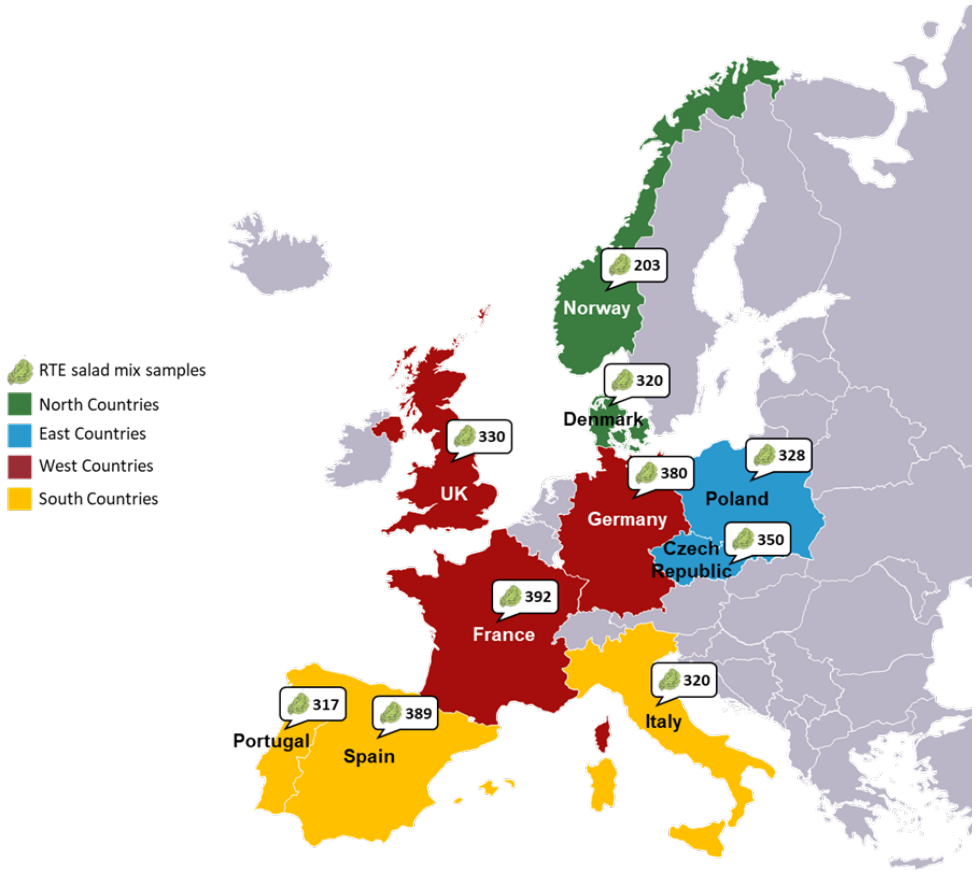
Multivariable analysis of risk factors for presence of Toxoplasma DNA in RTE salad

Variable	Category	OR	95% CI	P value
Sampling region	Northern Europe	1.00	-	-
	Eastern Europe	0.54	0.23-1.28	0.160
	Southern Europe	1.08	0.56-2.09	0.822
	Western Europe	3.31	1.82-6.04	<0.0001
Season	Autumn	1.0	-	-
	Winter	6.11	2.99-12.46	<0.0001
	Spring	4.19	2.00-8.79	<0.0001
	Summer	3.08	1.43-6.61	0.004

OR = Odd Ratio; N=3295

Variable	Category	OR	95% CI	P value
Packaging region	Northern Europe	1.00	-	-
	Eastern Europe	0.90	0.38-2.13	0.807
	Southern Europe	1.09	0.56-2.13	0.802
	Western Europe	3.27	1.80-5.96	<0.0001
Season	Autumn	1.0	-	-
	Winter	6.24	3.06-12.74	<0.0001
	Spring	4.50	2.15-9.43	<0.0001
	Summer	3.20	1.49-6.87	0.003

N=3017 (omitted “Europe” and “Unknown” categories for “Packaging region” in the analysis)



Conclusions

- A robust and standardized procedure was successfully implemented at European level to detect *T. gondii* oocyst contamination in fresh produce. Method sensitivity and specificity have been extensively evaluated!

- This is the first, largest and robust multicenter survey performed in EU to asses the occurrence of *T. gondii* DNA contamination in RTE mixed salad.

- Our approch and methodology will provide the bases for extended either either at European level or Nationally

- We show that a not negligible risk can be linked to RTE salad consumption being eventually source for human toxoplasmosis in Europe

- Our analysis suggests that winter and spring season as well as leaving in West Europe can be risk factors associated with T. gondii contamination with RTE salad

Study limitations

- No clear association between cultivation type, sample origin or site of sample packaging and positivity could emerge due to limitation in metadata availability

- Our study evaluate only *T. gondii* DNA presence but we have no visual (microscopy) evidence of *T. gondii* oocysts in the tested samples neiter prove of oocyst viability.

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