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Research Paper

Salmonella Prevalence in Raw Cocoa Beans and a Microbiological Risk Assessment to Evaluate the Impact of Cocoa Liquor Processing on the Reduction of *Salmonella*

Xingchen Zhao [1,6], Liesbeth Jacxsens [1], Vesela Tzeneva [2], Michiel Kokken [2], Anett Winkler [3], Cécile Vadier [4], Nicolau de Toledo [4], Tomasz Seliwiorstow [5], Mieke Uyttendaele [1,*]

[1] *Food Microbiology and Food Preservation Research Unit, Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium*
[2] *Olam Food Ingredients, Department Global Quality and Food Safety, Joan Muyskenweg 30T, 1114 AN Amsterdam, the Netherlands*
[3] *Cargill Holding (Germany) GmbH, Klaus-Bungert-Strasse 5b, 40468 Dusseldorf, Germany*
[4] *Barry-Callebaut France SAS, Rue de la Mécanique, 27400 Louviers, France*
[5] *Barry Callebaut Belgium NV, Aalstersestraat 122, 9280 Lebbeke-Wieze, Belgium*
[6] *Research Group for Food Microbiology and Hygiene, National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*

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ABSTRACT

Salmonella in raw cocoa beans (n = 870) from main sourcing areas over nine months was analyzed. It was detected in 71 (ca. 8.2%) samples, with a contamination level of 0.3–46 MPN/g except for one sample (4.1×10^4 CFU/g). Using prevalence and concentration data as input, the impact of thermal treatment in cocoa processing on the risk estimate of acquiring salmonellosis by a random Belgian chocolate consumer was calculated by a quantitative microbiological risk assessment (QMRA) approach. A modular process risk model from raw cocoa beans to cocoa liquor up to a hypothetical final product (70–90% dark chocolate tablet) was set up to understand changes in *Salmonella* concentrations following the production process. Different thermal treatments during bean or nib steam, nib roasting, or liquor sterilization (achieving a 0–6 log reduction of *Salmonella*) were simulated. Based on the generic FAO/WHO *Salmonella* dose–response model and the chocolate consumption data in Belgium, salmonellosis risk per serving and cases per year at population level were estimated. When a 5 log reduction of *Salmonella* was achieved, the estimated mean risk per serving was 3.35×10^{-8} (95% CI: 3.27×10^{-10}–1.59×10^{-7}), and estimated salmonellosis cases per year (11.7 million population) was 88 (95% CI: <1–418). The estimated mean risk per serving was 3.35×10^{-9} (95% CI: 3.27×10^{-11}–1.59×10^{-8}), and the estimated salmonellosis cases per year was 9 (95% CI: <1–42), for a 6 log reduction. The current QMRA model solely considered *Salmonella* reduction in a single-step thermal treatment in the cocoa process. Inactivation obtained during other process steps (e.g. grinding) might occur but was not considered. As the purpose was to use QMRA as a tool to evaluate the log reduction in the cocoa processing, no postcontamination from the processing environment and ingredients was included. A minimum of 5 log reduction of *Salmonella* in the single-step thermal treatment of cocoa process was considered to be adequate.

Salmonellosis caused by nontyphoidal *Salmonella enterica* is one of the most reported foodborne gastrointestinal infections in humans in the world. Foodborne outbreaks associated with *Salmonella* in chocolate products have been reported since the 1970s (Craven et al., 1975; D'Aoust, 1977; Gästrin et al., 1972) (Supplementary Table 1). Ingredients including cocoa beans have been implicated as the potential source of *Salmonella*-contaminated chocolate (Craven et al., 1975). Contamination occurring during the shipment and distribution of cocoa beans, from the processing environment, has also been referred to as the potential source of *Salmonella* in chocolate products (Supplementary Table 1) (EFSA & ECDC, 2022; Gill et al., 1983; Hockin, D'Aoust, et al., 1989; Kapperud et al., 1990). Besides, incidents due to the *Salmonella* contamination of additional ingredients (such as lecithin or milk powder) occurred sometimes (U.S. FDA, 2018; Whitworth Joe, 2022). It has been established that a low contamination level in chocolate products (e.g. ≤10 cells per 100 g) might be sufficient to cause foodborne disease (Hockin, D'Aoust, et al., 1989; Rao & Tamber, 2021). Such low contamination levels also pose a challenge

* Corresponding author.
E-mail address: mieke.uyttendaele@ugent.be (M. Uyttendaele).

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for monitoring and verification testing approaches. Even when established statistical sampling and validated testing procedures are followed, limitations towards the detection of such low contamination levels and time required to results exist (Jongenburger et al., 2015). Therefore, adequate process controls and food safety management systems in place are necessary to ensure the safety of products placed on the market (Zwietering et al., 2016).

Chocolate and other cocoa products are made from cocoa beans, collected/harvested from the cocoa fruit grown at the cocoa tree (*Theobroma cacao*). After the cocoa pods ripen and are harvested, the cocoa pods are opened, beans are fermented and then dried at the farms. The fermentation of cocoa beans is an important step in terms of the development of chocolate's flavor and aroma precursors (Moreno-Zambrano et al., 2018). Cocoa bean fermentation is a spontaneous on-farm process driven by a number of fungi and bacteria, thus leading to the final fermented cocoa beans of variable quality and microbial population (De Vuyst & Weckx, 2016; Ozturk & Young, 2017). Upon the completion of on-farm fermentation, the beans are dried either under the sun on the surface (e.g. wooden platforms, polypropylene sheets, concrete floor) or by mechanical means. After drying, the beans are sorted by hand to remove broken beans and other foreign bodies (Thompson et al., 2014; Winkler, 2023). All these steps might lead to the contamination of molds and Enterobacteriaceae (including *Salmonella*) via handling, surface and animal (e.g. insects, birds, and rodents) contact. Cocoa beans are mainly produced by smallholder farmers who sell their dried fermented cocoa beans to traders/distributors/cocoa processors (Winkler, 2023). Once collected in the central warehouses, beans are shipped to the destination of importers (e.g. Europe). Europe is the world's largest market for chocolate manufacturers and exporters and is also the world's largest importer of cocoa beans, with 56% of global imports (CBI, 2022). West Africa, Latin and South America, and Southeast Asia are the three main regions growing and supplying cocoa beans (CBI, 2022; Winkler, 2023).

As no data are publicly available on the prevalence and contamination levels of *Salmonella* in cocoa beans upon arrival in Europe, the **first objective** of the present study was to perform a survey which included sampling at the warehouses in Europe and testing for *Salmonella* in these cocoa beans imported from the main cocoa bean producing regions. **The second objective** of this study was, given the observed *Salmonella* prevalence and concentration in raw cocoa beans from the survey, to understand the impact of cocoa processing on the reduction of *Salmonella*. The heating steps (e.g. roasting, steam treatment) during processing in cocoa production are currently considered as critical control points (CCPs) leading to the reduction of pathogen contamination including *Salmonella* to an acceptable level (ICMSF, 2011).

In view of missing prevalence data on *Salmonella* in cocoa beans, the National Confectioners Association Chocolate Council (NCACC) recommends that a validated 4–5 log reduction of *Salmonella* is required during the roasting process (NCACC, 2011). This is in accordance with the Grocery Manufacturers of America (GMA) & other publications related to low−moisture foods (Schaffner et al., 2013). Considering vegetative microorganisms in general, which includes *Salmonella*, International Commission on Microbiological Specifications for Foods (ICMSF) refers to a 6 log reduction being achievable in cocoa processing (ICMSF, 2011).

Based on the results of prevalence and concentration of *Salmonella* in cocoa beans, a quantitative microbiological risk assessment (QMRA) on cocoa processing was performed to evaluate the degree of protection achieved by various target levels of *Salmonella* reduction at dedicated thermal treatment steps in cocoa processing. Therefore, a modular process risk model (MPRM) from raw cocoa beans to cocoa liquor and 70–90% dark chocolate tablets was set up, mimicking the various major processing steps in chocolate production. This MPRM included simulations of 0 to 6 log reductions during the chosen thermal processing step(s) (i.e. bean or nib steam, nib roasting, or liquor sterilization). The QMRA assumed that the initial contamination of raw cocoa beans is the main driver for *Salmonella* introduction in the cocoa liquor. The heat treatment (e.g. roasting, steam treatment) is the only step where *Salmonella* reduction occurs, and no postcontamination from the processing environment or ingredients occurs. The outcome of the QMRA, the estimated risk per serving and annual cases of human salmonellosis associated with the hypothetical dark chocolate tablet consumption by the Belgian population (as an example for the European population), was compared to other QMRA studies and recognized degrees of protection that have been established over time by consensus or by regulation. A reasonable guideline for drinking water regarding the waterborne disease burden from the U.S. EPA, one illness per 10,000 individuals in a given year (U.S. EPA, 1989), was considered a benchmark that could be used in the present study.

Materials and methods

Survey of Salmonella in raw cocoa beans imported to Europe

Sampling design

In total, 870 samples of raw cocoa beans from warehouses in Europe were collected by three cocoa manufacturers during the period of November 2022–July 2023 (season of production). Approximately, 80% of the samples came from the main crop in the period of November 2022–May 2023, and 20% of the samples were from the mid-crop in the period of May 2023–July 2023. Around 90% of the samples originated from the five biggest cocoa bean−producing countries (Ivory Coast, Ghana, Nigeria, Cameroon, and Ecuador), and the other 10% of the samples were from other origins.

Detection of Salmonella

Whole bean samples (approximately 1 kg of each) were collected and delivered to a commercial food diagnostic service lab (Eurofins laboratoire de Microbiologie Ouest, Nantes, France) to detect *Salmonella*, and if *Salmonella* was detected, the number of *Salmonella* was estimated. The whole bean samples were hammer-crushed in the laboratory before analysis, and the detection methods were illustrated in Supplementary Fig. 1. A 375-g portion of each crushed raw cocoa bean sample was analyzed for *Salmonella* using the commercial BAC*Gene Salmonella* PCR method (Gold Standard Diagnostics). It is a qualitative real-time PCR assay that utilizes unique primer and probe components for highly specific detection of genes unique to *Salmonella* spp. The PCR-based *Salmonella* detection protocol has been certified by AOAC Certification as an alternative method for the ISO 6579-1: 2017 classical culture method detection of *Salmonella,* according to ISO 16140-2:2016 method validation protocol (ISO 6579-1:2017; ISO 16140-2:2016). Its performance characteristics were also verified on cocoa beans in the service laboratory according to ISO 16140-3: 2021 protocol (ISO 16140-3: 2021). Briefly, 375 g cocoa bean samples were preenriched in 3375 mL UHT skimmed milk to overcome inhibitory effects caused by polyphenols from cocoa, supplemented with 18 mL Tween 80 and 0.068 g Brilliant Green (BG) to inhibit the growth of Gram-positive bacteria (Jasson et al., 2011). After 24-h preenrichment at 37 °C, DNA extraction was performed using BAC*Gene Salmonella* kit (Eurofins GeneScan Technologies, Freiburg, Germany). Presumptive *Salmonella* was detected using a direct multiplex qualitative real-time PCR (qPCR) with a positive signal that required confirmation. The amplified fragments are detected via FAM or R6G fluorescence-labeled hybridization probes quenched by a nonfluorescent quencher. An internal positive control (IPC) is included in the MasterMix, and the IPC detection indicates the proper functioning of the qPCR. In case of positive *Salmonella* PCR detection, a confirmation step was performed by subculture of 100 µL from the enrichment broth in 9.9 mL RVS broth (incubation ca. 24 h at 41.5 °C) and 1 ml in 9 ml

MKTTn broth (ca. 24 h at 37 °C) followed by streaking onto Xylose Lysine Deoxycholate (XLD) agar and a chromogenic agar (Brilliance *Salmonella* agar) followed by latex test on isolated typical colonies without a purification step and biochemical confirmation using commercial galleries applicable for biochemical confirmation of *Salmonella* according to ISO 6579-1 protocol (ISO 6579-1:2017). The resulting limit of detection$_{100}$ (LOD_{100}) of the qPCR assay corresponds to the estimated number of 3 CFU per 375 g, i.e. 0.008 CFU/g. LOD_{100} means when the PCR result is negative, *Salmonella* counts will always be below (<) 3 CFU/375 g

Enumeration of Salmonella and data treatment

Samples with a detection of *Salmonella* were subjected to the nine-tube most-probable-number (MPN) tests (ISO 7218:2007/Amd 1:2013). In brief, 50 g crushed beans were diluted in 450 mL milk with Tween 80 and BG, resulting in a 10^{-1} dilution (S1). Then, 100 mL, 10 mL, and 1 mL of S1 (corresponding to 10 g, 1 g and 0.1 g samples respectively) with three replicates of each were incubated at 37 °C overnight and tested again using the BAC*Gene* PCR protocol. The MPN value was determined based on the number of positive tubes in each of the three sets and the standard MPN table. The theoretical limit of quantitation (LOQ) equals 0.3 MPN/g (ISO 7218:2007/Amd 1:2013; Jarvis et al., 2010). The qPCR assay described in Section '**Detection of *Salmonella***' was performed on the 9 tubes (or bags) to confirm the presence of *Salmonella*. The confirmation tests were not performed again here as the confirmation had been performed in Section '**Detection of *Salmonella***'.

Once a sample tested positive for all nine MPN tubes (>110 MPN/g), this sample (the rest portion from 1 kg beans) was delivered to the Food Microbiology and Food Preservation Research Unit (LFMFP) at Ghent University for the enumeration of *Salmonella* using direct plating techniques. Briefly, 25 g crushed beans were 10-fold diluted with buffered peptone water (BPW) and homogenized for 1 min in a filtered stomacher bag. A serial 10-fold dilution was made in peptone physiological solution (PPS; 1 g/L peptone + 8.5 g/L NaCl) and spread-plated on XLD agar top-layered with 9 mL trypticase soy agar (TSA). It was aimed to recover the sublethal−injured *Salmonella* cells (Kang & Fung, 2000).

Four classes of *Salmonella* concentrations were obtained and illustrated in Supplementary Fig. 1: (1) left-censored results (<LOD_{100}): either not detected by qPCR or detected by qPCR but not confirmed as *Salmonella*; (2) interval-censored results (≥LOD_{100}, < LOQ): detected by qPCR and confirmed as *Salmonella* but having a 0–0–0 MPN dilution pattern; (3) MPN quantitative results (0.3–110 MPN/g): detected by qPCR and confirmed as *Salmonella*, and an MPN dilution pattern providing an estimated count; (4) colony counting quantitative results (≥100 CFU/g): detected by qPCR and confirmed as *Salmonella* but providing a 3–3–3 MPN dilution pattern which initiated a direct plate count for *Salmonella*.

Quantitative microbiological risk assessment (QMRA)

Modular process risk model (MPRM) from raw cocoa bean to dark chocolate

Cocoa liquor production process

Four commonly used/general cocoa liquor production processes distinct by their thermal treatment step are shown in Fig. 1. The production processes start with raw whole beans with final moisture ≤8% in European warehouses. Beans are stored in the warehouse until transported to the factory for further processing. Precleaning to remove foreign bodies such as stones and dust is undertaken. After the precleaning and short storage in silos, different process flows can be chosen based on different thermal treatments: 'bean heat treatment', 'nib heat treatment', or 'liquor heat treatment'.

For the bean heat processing, steam treatment of cocoa beans as the CCP step is introduced (Fig. 1a). Subsequently, beans are dried (to reduce the moisture after steam treatment) and roasted to achieve the desired flavors, then the beans are broken, and shells and nibs are separated. Nibs are further ground into cocoa liquor. For the nib heat processing, nibs are first obtained by bean breaking and winnowing. Depending on the process, nibs may undergo alkalization before/after or together with the CCP step to achieve a distinct dark color (Winkler, 2023). Two different thermal treatments as the CCP step can be chosen, including nib steam (Fig. 1b) and nib roasting supplemented with water to achieve desired qualities and flavor profiles (Fig. 1c). After drying/roasting, nibs are ground into cocoa liquor and stored. For the liquor heat processing (Fig. 1d), the CCP step is undertaken on the cocoa liquor (after the roasted nib grinding). The production processes above all end up with cocoa liquor/mass stored in tanks under appropriate storage conditions.

Module setup and general assumptions

Three processes were summarized and used in the MPRM resulting (1) bean heat process (Fig. 1 flow a), (2) nib heat process (Fig. 1, integrating flow b + c), and (3) liquor heat process (Fig. 1 flow d). The production steps (Section '**Cocoa liquor production process**' and Fig. 1a,–d) were translated into nine modules (Table 1). Two extra modules were added to cover until the example of final product, which was 70–90% dark chocolate tablet. Optional production steps (i.e. preheating and alkalization) were not included in the MPRM. General assumptions were made for the MPRM taking into account the purpose of the QMRA as a tool to evaluate the log reduction in the cocoa processing: (1) no environmental postcontamination of *Salmonella*; (2) 0–6 log reductions of *Salmonella* were considered at the single step (CCP) thermal treatment; (3) the considered CCP step was the only inactivation step of *Salmonella*, although further reduction of the microbial load could also be achieved due to time/temperature conditions at other processing steps such as 'roasting/drying'; they were here considered as technological steps for flavor and quality properties only; (4) a hypothetical dark chocolate tablet containing 70–90% cocoa liquor was assumed to be the final product, and no *Salmonella* contamination was assumed to occur by any other ingredients in this tablet. Model inputs including variables, distributions, and calculating formulas are provided in Table 3. Due to some knowledge or data gaps in each module, specific assumptions were made and are outlined to allow the completion of the quantitative model (Table 1). The module setup and assumptions were developed with the support of industry partners involved in this study. They provided their best estimation for the parameters in the production process such as the unit size, and the data source was shown as 'industry information' in Table 2.

In each module, presented in Table 1, the distribution of numbers of *Salmonella* in the given process unit was estimated. A unit represents cocoa beans/nibs/liquor in a lot, batch, tank, or tablet; thus, the unit size (weight) can be different in different steps of the production process (Table 1).

Module description

Module 1: Raw whole beans.

The results from the prevalence study described in Section '*Survey of Salmonella in raw cocoa beans imported to Europe*' were considered as the initial input *Salmonella* concentration in raw cocoa beans and were taken as the starting point for the MPRM. It was assumed that *Salmonella* was absent in raw cocoa beans when *Salmonella* was not detected. Thus, *Salmonella* concentration in the contaminated beans as the model input consisted of interval censored (≥LOD_{100}, <LOQ) and quantitative data (≥LOQ), including all samples showing positive results for *Salmonella* detection (n = 71). A maximum likelihood-based approach was used and implemented in R version 4.3.1 (https://www.r-project.org/) using R package 'fitdistrplus' (Delignette-Muller & Dutang, 2015) to estimate the parameters of a

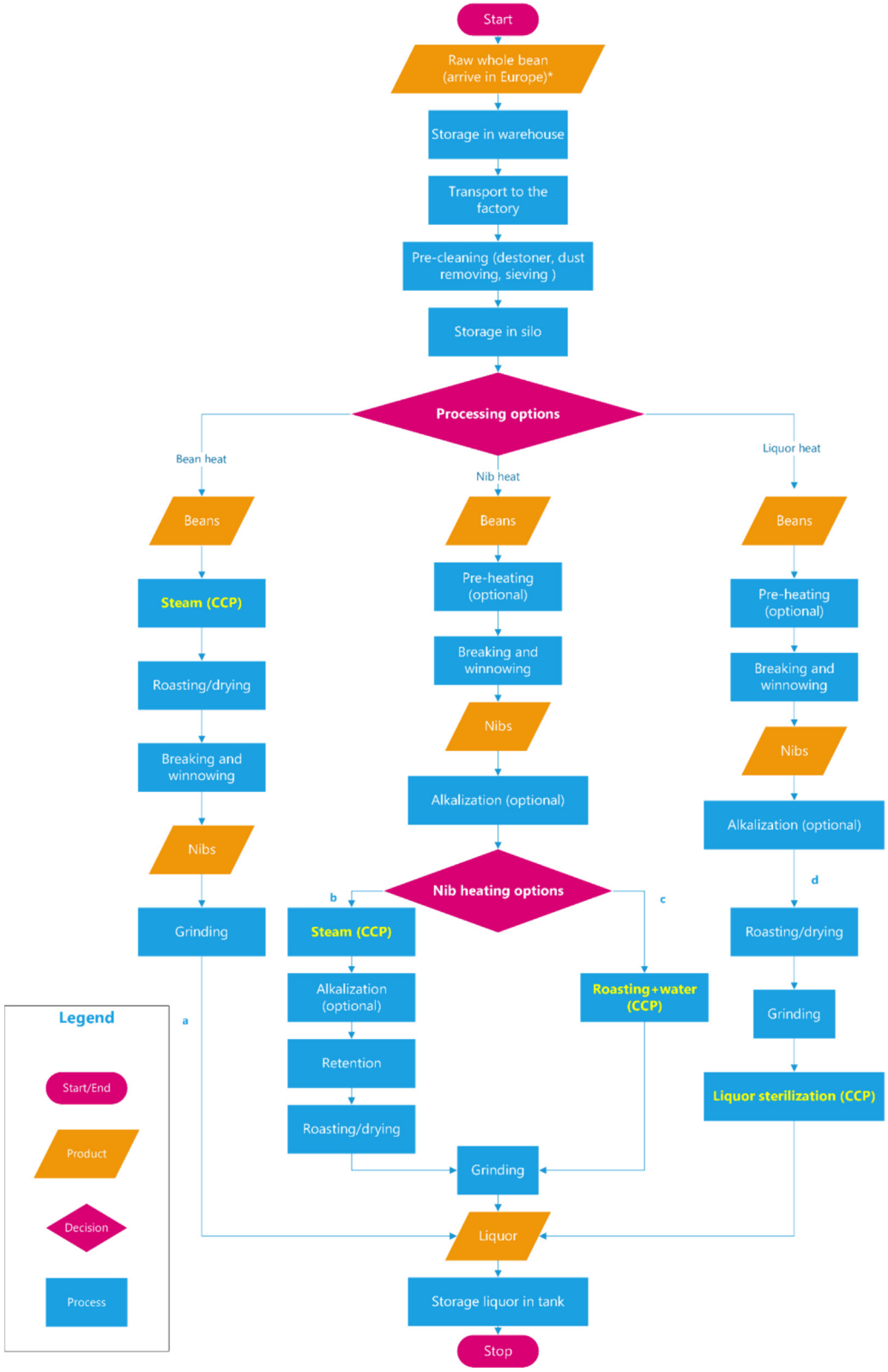

Figure 1. Flow chart of production processes of cocoa liquor. (a) bean heat process, (b & c) nib heat process with either steam or roasting, (d) liquor heat process. The major thermal step is indicated as CCP. *The step where the prevalence study was established.

parametric distribution from this dataset including censored data. Data were first transformed into log (log_{10}) values; then, *Salmonella* concentration data were fitted to normal and logistic distributions (Busschaert et al., 2010; Delignette-Muller & Dutang, 2015; Farakos, Pouillot, Johnson, Spungen, Son, Anderson, & Van Doren, 2017; Pouillot & Delignette-Muller, 2010; Williams et al., 2013). The quality of fit was evaluated using Akaike and Schwarz's Bayesian information criteria (AIC and BIC) and the goodness-of-fit cumulative distribution

Table 1
Modules, their corresponding processing steps, and assumptions in MPRM of cocoa liquor production, including bean heat, nib heat, and liquor heat process (refer to Fig. 1). Quantitative impacts refer to the tables and Fig. 3 below for outputs

Module	Module code	Module description	Assumption	Effect on		
				C (log CFU/g)[a]	Unit size (ton)	N_{total} (in the unit)[b]
MPRM1: Bean heat process (Fig. 1a)						
1	B0	Raw whole beans (sampling)	Input: 71 *Salmonella* positive sampling results from 3 companies without considering different origins.	/	/	/
2	St	Storage in the warehouse + transport to the factory	Worst case scenario: no reductions of *Salmonella* concentration. Unit size reduces.	=	−	−
3	Cl	Precleaning (remove foreign bodies)	No change of *Salmonella* concentration, weight loss due to cleaning. Unit size reduces.	=	−	−
4	S2	Storage in the silo	1–3 day storage; no change of *Salmonella* concentration and unit size.	=	=	=
5	H1	Bean steam (CCP)	Inactivation step: 0 to 6 log reduction treatment levels; Unit size reduces into 1 ton (bean partitioning).	−	−	−
6	Rd	Roasting/drying*	Due to the total weight loss of moisture, N_{total} of *Salmonella* in the unit won't change, but the concentration of *Salmonella* increases. Unit size reduces.	+	−	=
7	Re	Breaking and winnowing into nibs*	Worst case scenario: no reductions of *Salmonella* concentration. Weight loss due to shell removal, thus unit size reduces.	=	−	−
8	Gr	Grinding into liquor*	Worst case scenario: no reductions of *Salmonella* concentration. No change of unit size and weight.	=	=	=
9	S3	Storage liquor in tank	Worst case scenario: no reductions of *Salmonella* concentration. Mix batches of liquor into the size of the tank, thus unit size increases.	=	+	+
10	P	Further processes + mixing with other ingredients	Worst case scenario: no reductions of *Salmonella* concentration in liquor. Recontamination has not been accounted for. Thus, *Salmonella* concentration decreases by mixing 70–90% liquor with other ingredients. Unit size increases by adding other ingredients.	−	+	=
11	EP	Final product = >70–90% dark chocolate tablet	No change of *Salmonella* concentration. Unit size reduces (partitioning into package)	=	−	−
MPRM2: Nib heat process (Fig. 1b & c)						
1	B0	Raw whole beans (sampling)	Same as MPRM1 Module 1	/	/	/
2	St	Storage in the warehouse + transport to the factory	Same as MPRM1 Module 2	=	−	−
3	Cl	Precleaning (remove foreign bodies)	Same as MPRM1 Module 3	=	−	−
4	S2	Storage in the silo	Same as MPRM1 Module 4	=	=	=
5	Re	Breaking and winnowing into nibs*	Same as MPRM1 Module 7	=	−	−
6	H2	Nib steam (CCP) **or** nib roasting supplied with water (CCP)	Inactivation step: 0 to 6 log reduction treatment levels. Unit size reduces into 1 ton (nib partitioning).	−	−	−
7	Rd	Retention + roasting/drying*	Same as MPRM1 Module 6	+	−	=
8	Gr	Grinding into liquor*	Same as MPRM1 Module 8	=	=	=
9	S3	Storage liquor in tank	Same as MPRM1 Module 9	=	+	+
10	P	Further processes + mixing with other ingredients	Same as MPRM1 Module 10	−	+	=
11	EP	Final product = >70–90% dark chocolate tablet	Same as MPRM1 Module 11	=	−	−
MPRM3: Liquor heat process (Fig. 1d)						
1	B0	Raw whole beans (sampling)	Same as MPRM1 Module 1	/	/	/
2	St	Storage in the warehouse + transport to the factory	Same as MPRM1 Module 2	=	−	−
3	Cl	Precleaning (remove foreign bodies)	Same as MPRM1 Module 3	=	−	−
4	S2	Storage in the silo	Same as MPRM1 Module 4	=	=	=
5	Re	Breaking and winnowing into nibs*	Same as MPRM1 Module 7	=	−	−
6	Rd	Roasting/drying*	Same as MPRM1 Module 6	+	−	=
7	Gr	Grinding into liquor*	Same as MPRM1 Module 8	=	=	=
8	H3	Liquor sterilization (CCP)	Inactivation step: 0–6 log reduction treatment levels. Unit size reduces to 1 ton (liquor partitioning)	−	−	−
9	S3	Storage liquor in tank	Same as MPRM1 Module 9	=	+	+
10	P	Further processes + mixing with other ingredients	Same as MPRM1 Module 10	−	+	=
11	EP	Final product = >70–90% dark chocolate tablet	Same as MPRM1 Module 11	=	−	−

(−) decrease, (+) increase, and (=) no change in this process, (/) not relevant.

[a] Concentration of *Salmonella* in the products (beans, nibs, liquor, or chocolates). General assumption of the unit of *Salmonella* quantitative data: MPN/g = CFU/g.

[b] Total number of *Salmonella* present in the unit.

* Considered as technological steps for flavor and quality properties, potential reductions due to the heat generated in those processing steps have not been considered in the model.

Table 2
Variables used in three MPRMs from raw cocoa bean to cocoa liquor and dark chocolate tablet

Variable	Description	Value/distribution/formula	Unit	Source
MPRM1: Bean heat process (Fig. 1a)				
C_B0	*Salmonella* concentration contaminated in raw cocoa beans arriving in Europe based on the survey	RiskLogistic (−0.8669374; 0.3577331)	log CFU/g	Calculated
U_B0	Unit size (weight) of raw cocoa beans arrived in European warehouses	RiskPert (100;5,050;10,000)	ton	Industry information
U_St	Unit size (weight) of raw cocoa beans entered the cocoa processing factory	RiskPert (20;510;1,000)	ton	Industry information
U_Cl	Weight of raw cocoa beans after cleaning	U_St*RiskPert (0.95;0.97;0.99)	ton	Industry information
C_H1	*Salmonella* concentration after bean steam, assumed 0–6 log reductions	C_B0- x (x = 0, 1, 2, 3, 4, 5, or 6)	log CFU/g	Assumption
U_H1	Unit size of steamer during heat inactivation	1	ton	Industry information
U_Rd	Weight of cocoa beans after roasting/drying	U_H1*RiskPert (0.93;0.935;0.94)	ton	Industry information
C_Rd	*Salmonella* concentration after roasting/drying	Log10 (10^{C_H1}*U_H1*10^6/(U_Rd*10^6))	log CFU/g	Calculated
U_Re	Weight of nibs after removing shells	U_Rd*RiskPert (0.87;0.92;0.93)	ton	Industry information
U_S3	Unit size of tank for liquor storage	RiskPert (20;25;50)	ton	Industry information
U_P	Weight of cocoa liquor mixed with other ingredients	U_S3/RiskPert (0.7;0.8;0.9)	ton	Assumption
C_P	*Salmonella* concentration after mixing with other ingredients	Log10 (10^{C_Rd}*U_S3*10^6/(U_P*10^6))	log CFU/g	Calculated
MPRM2: Nib heat process (Fig. 1b & c)				
C_B0	*Salmonella* concentration contaminated in raw cocoa beans arriving in Europe based on the survey	Same formula as C_B0 in MPRM1	log CFU/g	Calculated
U_B0	Unit size (weight) of raw cocoa beans arrived in European warehouses	Same formula as U_B0 in MPRM1	ton	Industry information
U_St	Unit size (weight) of raw cocoa beans entered the cocoa processing factory	Same formula as U_St in MPRM1	ton	Industry information
U_Cl	Weight of raw cocoa beans after cleaning	Same formula as U_Cl in MPRM1	ton	Industry information
U_Re	Weight of nibs after removing shells	U_Cl* RiskPert (0.87;0.92;0.93)	ton	Industry information
C_H2	*Salmonella* concentration after nib steam or nib roasting, assumed 0–6 log reductions	Same formula as C_H1 in MPRM1	log CFU/g	Assumption
U_H2	Unit size of steamer or roaster during heat inactivation	1	ton	Industry information
U_Rd	Weight of cocoa beans after roasting/drying	U_H2*RiskPert (0.93;0.935;0.94)	ton	Industry information
C_Rd	*Salmonella* concentration after roasting/drying	Log10 (10^{C_H2}*U_H2*10^6/(U_Rd*10^6))	log CFU/g	Calculated
U_S3	Unit size of tank for liquor storage	Same formula as U_S3 in MPRM1	ton	Industry information
U_P	Weight of cocoa liquor mixed with other ingredients	Same formula as U_P in MPRM1	ton	Assumption
C_P	*Salmonella* concentration after mixing with other ingredients	Same formula as C_P in MPRM1	log CFU/g	Calculated
MPRM3: Liquor heat process (Fig. 1d)				
C_B0	*Salmonella* concentration contaminated in raw cocoa beans arriving in Europe based on the survey	Same formula as C_B0 in MPRM1	log CFU/g	Calculated
U_B0	Unit size (weight) of raw cocoa beans arrived in European warehouses	Same formula as U_B0 in MPRM1	ton	Industry information
U_St	Unit size (weight) of raw cocoa beans entered the cocoa processing factory	Same formula as U_St in MPRM1	ton	Industry information
U_Cl	Weight of raw cocoa beans after cleaning	Same formula as U_Cl in MPRM1	ton	Industry information
U_Re	Weight of nibs after removing shells	Same formula as U_Re in MPRM2	ton	Industry information
U_Rd	Weight of cocoa beans after roasting/drying	U_Re*RiskPert (0.93;0.935;0.94)	ton	Industry information
C_Rd	*Salmonella* concentration after roasting/drying	Log10 (10^{C_B0}*U_Re*10^6/(U_Rd*10^6))	log CFU/g	Calculated
C_H3	*Salmonella* concentration after liquor sterilization, assumed 0–6 log reductions	C_Rd- x (x = 0, 1, 2, 3, 4, 5, or 6)	log CFU/g	Assumption
U_H3	Unit size of liquor sterilizer during heat inactivation	1	ton	Industry information
U_S3	Unit size of tank for liquor storage	Same formula as U_S3 in MPRM1	ton	Industry information
U_P	Weight of cocoa liquor mixed with other ingredients	Same formula as U_P in MPRM1	ton	Assumption
C_P	*Salmonella* concentration after mixing with other ingredients	Log10 (10^{C_H3}*U_S3*10^6/(U_P*10^6))	log CFU/g	Calculated

function (CDF) plot (Supplementary Fig. 2). Besides, the major statistics of the estimated distribution were also checked by comparing with the original dataset. Based on the criteria mentioned above, the logistic distribution was defined as the best-fit distribution. The estimated parameters α (location) and β (scale) were used to define the logistic distribution in the @Risk software (version 8 Palisade Corporation, US) for the MPRM. To prevent the model from simulating extremely high *Salmonella* concentrations in the raw cocoa beans, the distributions were truncated on the right side at 8 log CFU/g based on expert opinion (Table 2). The estimated lot size of cocoa beans in the warehouse is 100–10,000 tons and follows a Pert distribution (minimum = 100 tons, most likely = 5,050 tons, maximum = 10,000 tons; Table 2).

Module 2–4: Storage, transportation, precleaning, and intermediate storage. No data is currently available for the behavior of *Salmonella* in cocoa beans during storage and transportation. As such, no *Salmonella* reduction was assumed during the cocoa bean storage and transportation within Europe. The unit size of beans entering the factory for one batch was estimated to follow a Pert distribution with a minimum of 20 tons, most likely 510 tons, and a maximum of 1,000 tons. Precleaning to remove foreign bodies can be done either in the warehouse or the factory, and in this study, it was assumed to be done in the factory. The process of precleaning causes approximately 1–5% weight loss which results in 95–99% (most likely 97%) weight remaining of the beans. Due to short bean storage in the factory (ca. 1–3 days), no *Salmonella* reduction and unit size changes were considered (Table 1).

Module 5–9: Bean, nib, or liquor heat process to obtain cocoa liquor. Seven treatment levels including 0–6 log reductions of *Salmonella* were given in the MPRM for all three processes. For all three processes, the representative unit size was 1 ton for the steamer, roaster, or liquor heating equipment, which resulted in the partitioning of beans, nibs, or liquors from the previous module. Roasting/drying was involved in all three processes, but it could take place at different processing steps on either beans or nibs (Fig. 1). It was assumed that a 6–7% (most likely 6.5%) weight loss occurred due to

Table 3
Variables used in hazard characterization, exposure assessment and risk characterization for *Salmonella* in dark chocolate tablet

Variable	Description	Value/ distribution/formula	Unit	Source
U_S	Serving size of dark chocolate	RiskPert (18;28;40)	g	EFSA (2022)
D_S	*Salmonella* dose per contaminated serving	10^C_P*U_S	CFU	Calculated
α	Dose-response model parameter α	RiskPert (0.0763;0.0324;0.2274)	/	FAO/WHO (2022)
β	Dose-response model parameter β	RiskPert (38.49;51.45;57.96)	/	FAO/WHO (2022)
P_{ill}	Probability of illness due to the *Salmonella* exposure per single dose	1 − (1 + D_S/β)^-α	/	FAO/WHO (2022); Calculated
P	Probability of contaminated servings	RiskBeta (72;800)	/	Data
Rs	Risk per serving	Pill*P	/	Farakos et al. (2017); FAO/WHO (2022); Calculated
Ef	Exposure frequency per consumer per year	365	Serving	Assumption – worst-case
Ry	Annual risk per consumer	Rs*Ef	/	FAO/WHO (2022); Calculated
BEp	Belgian population	11,697,557	Inhabitant	STATBEL (2023)
%CS	Percentage of Belgian dark chocolate consumers	RiskPert (34;63;87)	%	EFSA (2022); Assumption
N_CS	Number of Belgian dark chocolate consumers	BEp*%CS	Person	Calculated
Ncpy	Annual human salmonellosis cases in Belgium due to the consumption of dark chocolate tablet	Ry*N_CS	Cases per year	FAO/WHO (2022); Calculated
Ncpppy	Belgian human salmonellosis cases per person per year due to the consumption of dark chocolate tablet	Ncpy/BEp	Cases per person per year	Calculated

"/" means not relevant.

the moisture reduction. The total number of *Salmonella* in the unit will remain unchanged, but the concentration of *Salmonella* will increase due to the moisture reduction. During breaking and winnowing, ca. 7–13% (most likely 8%) weight loss was considered because of the shell removal. The Codex requirement of a maximum 1.75% cocoa shell and germ was calculated on an alkali − free basis (FAO/WHO, 2022). It was also assumed that *Salmonella* cells were equally distributed in the shells and nibs of contaminated beans, as the worst-case scenario. Thus, the shell removal would not change the *Salmonella* concentration from beans to nibs. During the process of grinding, no reduction of *Salmonella* and change of the unit size were considered. Afterward, batches of cocoa liquor were mixed into storage tanks with a bigger size ranging from 20 tons to 50 tons (most likely scenario being 25 tons) (Table 2).

Module 10–11: Further processes to the final product. The estimated *Salmonella* concentration in 70–90% dark chocolate tablets is applied as the final estimate in this MPRM. It was assumed that *Salmonella* contamination only came from cocoa liquor and no postcontamination of *Salmonella* from the other ingredients (e.g. milk powder) or environment was considered. Thus, the *Salmonella* concentration was diluted by mixing approximately 70–90% (most likely 80%) cocoa liquor with other ingredients. By adding other ingredients, the unit size of the total chocolate mass was assumed to increase. No further *Salmonella* reduction was assumed in the following processes (e.g. grinding, conching) to the dark chocolate as the worst case.

Hazard characterization, exposure assessment, and risk characterization

Hazard characterization and exposure assessment. The chocolate consumption data of Belgian population were used in the exposure assessment of this QMRA, as Belgium is one of the most chocolate − producing and consuming countries in Europe. A serving size of dark chocolate was defined as 18–40 g (minimum–maximum) with a mean of 28 g based on Belgian consumers' chronic food consumption data (g/day) under the category of 'Confectionery including chocolate' (EFSA, 2022). The consumption data of all investigated population groups including toddlers, other children, adolescents, adults, elderly, and very elderly were taken into account (Supplementary Table 2). These values were applied to a Pert distribution (Table 3). Thus, *Salmonella* dose per contaminated serving of dark chocolate was calculated by multiplying the serving size by the calculated *Salmonella* concentration in 70–90% dark chocolate.

The beta-Poisson model (Table 3) proposed by World Health Organization, Food and Agriculture Organization of the United Nations (FAO/WHO, 2002) was used in this QMRA to describe the dose–response relationship between the ingested dose of generic *Salmonella* spp. and the probability of human illness (salmonellosis). The dose–response parameters (α and β, Table 3) derived from FAO and WHO (2002) were modeled by Pert distribution.

Risk characterization. The exposure as outcome of the QMRA was calculated including the 71 *Salmonella*-contaminated raw cocoa bean samples as the model input (Section '**Module description**' & Fig. 2), resulting in the *Salmonella* dose per contaminated serving. Therefore, to estimate the risk per serving (as a randomly selected serving including both contaminated and noncontaminated servings), the probability of contaminated servings should be considered (Fig. 2). The probability of contaminated servings was assumed to be equivalent to the prevalence of *Salmonella*-contaminated raw cocoa beans (i.e. 8.2%) obtained from the prevalence study described in Section '*Survey of Salmonella in raw cocoa beans imported to Europe*', following a Beta distribution with parameters $\alpha = x + 1 = 71$ and $\beta = n - x + 1 = 800$ (Table 3), as no other routes of contamination (no environmental contamination nor postcontamination by ingredients) were assumed. The estimated risk per serving was calculated by multiplying the estimated risk per contaminated serving and the probability of contaminated servings (FAO/WHO, 2002; Farakos, Pouillot, Johnson, Spungen, Son, Anderson, & Van Doren, 2017). The annual risk per consumer was calculated by multiplying the estimated risk per serving with multiple exposures (servings) consumed in a year (FAO/WHO, 2002). It was assumed that the risk posed by a single exposure (serving) was independent of any other exposure (serving). The worst case of exposure frequency per consumer per year (days of dark chocolate consumption) was given as 365 which represented that the Belgian consumer was assumed to eat dark chocolate every day (Table 3 & Fig. 2).

In addition to estimating risk per serving and annual risk per consumer, the annual human salmonellosis cases in Belgium due to the consumption of dark chocolate were also estimated by considering the annual risk per consumer (the probability of illness per consumer per year) and number of chocolate consumers in Belgium (Table 3). It has been reported that ca. 34–87% (mean = 63%) of the Belgian participants in the national food consumption survey were consumers of confectionery including chocolate (EFSA, 2022), considering all population groups. Therefore, it was assumed that 34–87% (most likely: 63%) of the Belgian population (11,697,557 inhabitants) were dark chocolate consumers (STATBEL, 2023). As the final output of the the-

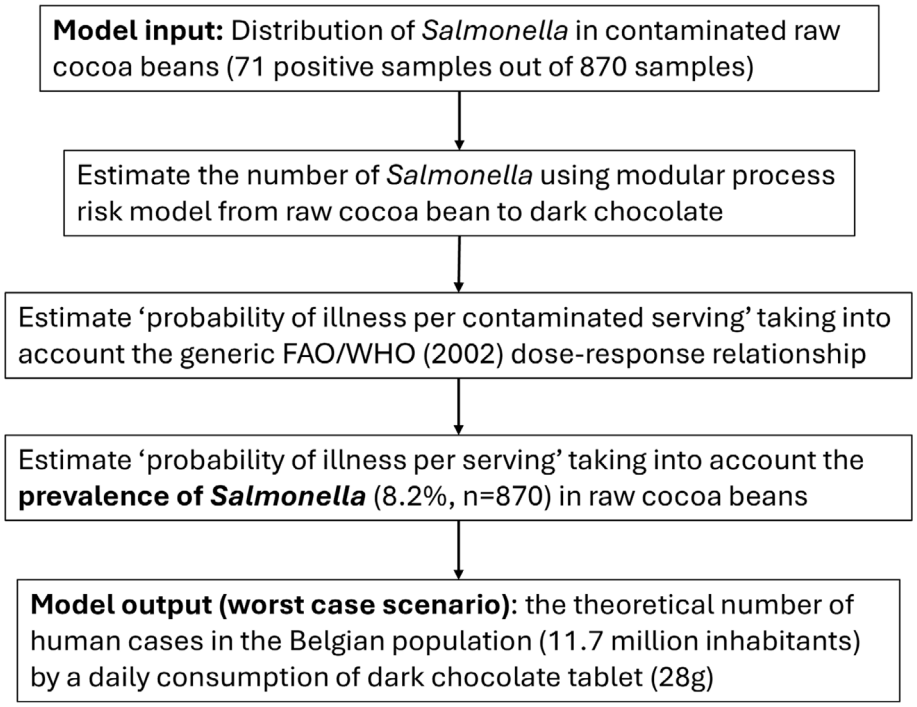

Figure 2. Quantitative Microbial Risk Assessment (QMRA) model illustration in this study.

oretical QMRA, Belgian human salmonellosis cases per person per year due to the consumption of dark chocolate were estimated by annual human salmonellosis cases in Belgium divided by the Belgian population (Table 3).

Modeling software and simulations

The model was run 10^5 iterations for each simulation using Latin Hypercube sampling in @Risk software (version 8 Palisade Corporation, US). Bean heat process was selected, and simulation stability was controlled using (1) 10^5 iterations with 1 simulation, (2) 10^5 iterations with 2 simulations, and (3) 10^6 iterations with 1 simulation. This number (10^5 iterations with 1 simulation) was chosen as a compromise considering both the need for accuracy and available computing power.

Sensitivity analysis

The sensitivity analysis was developed using the correlation methodology by calculating the Spearman rank correlation coefficient (between −1 and 1). Spearman rank correlation coefficient was checked using @risk software to determine which of the input distributions had the largest effect on the risk per serving being the output in the model.

Results

Prevalence and concentration of Salmonella in raw cocoa beans arriving in Europe

A prevalence of 8.2% (71/870) *Salmonella* contaminated raw cocoa beans was found in this study, and a Beta distribution was fitted as mentioned in Section '**Risk characterization**', resulting in the 95% confidence interval (CI) ranging from 6.4% to 10.0%. In tested 870 raw cocoa bean samples, *Salmonella* was not detected in 800 samples, resulting in the left-censored (<0.008) data of *Salmonella* concentrations (Supplementary Fig. 1 and Supplementary Table 3). In 71 samples, qPCR assay gave positive results (presumptive detection of *Salmonella*). In 51 samples, the MPN dilution pattern was 0–0–0, resulting in the interval-censored *Salmonella* concentrations (Supplementary Fig. 1 and Supplementary Table 3). In 19 *Salmonella*-positive samples, 0.3 to >110 MPN/g *Salmonella* were measured, and the one sample with >110 MPN/g *Salmonella* was further subjected to enumeration using XLD top-layered TSA which led to the concentration of 4.1×10^4 CFU/g (Supplementary Tables 3 and 4). The sampling was conducted over nine months and included beans from major cocoa − producing areas.

Theoretical QMRA output

Exposure assessment of Salmonella in dark chocolate

The theoretical QMRA showed throughout the three simulated processes (Fig. 3a,–c), the estimated *Salmonella* numbers show a 10-fold decrease by each additional log reduction during the CCP step (bean heat: H1, nib heat: H2 or liquor heat: H3), to the final product (Fig. 3 and Supplementary Table 5). Fig. 3 reveals the change (increase or decrease) of *Salmonella* levels between modules (steps in process) and apart from the CCP step, changes in *Salmonella* levels are occurring due to the change of unit size by mixing or partitioning (as no growth nor postcontamination is assumed). For instance, the increase of *Salmonella* numbers from Module Gr (grinding into liquor) to S3 (storage in the liquor tank) is caused by mixing smaller sizes of liquor after grinding into a bigger size of liquor tank for storage (Fig. 3), and not because of the growth of *Salmonella* in cocoa liquor.

Because the model outputs of *Salmonella* numbers in module S3 (liquor) and module EP (dark chocolate) are the same for all three simulated models, only the outputs from the bean heat process are shown in Supplementary Table 5 as an example. When a 4 log reduction is achieved, 1850 (mean; 95% CI: 19.3–8550) CFU/28-ton (mean) cocoa liquor and 1.49×10^{-3} (mean; 95% CI: 1.57×10^{-5}–6.90×10^{-3}) CFU/28-g (mean) 70–90% dark chocolate tablet of *Salmonella* are estimated, respectively (Supplementary Table 5). If a 5 log reduction is reached, 185 (mean; 95% CI: 1.93–855) CFU/28-ton (mean) cocoa liquor and 1.49×10^{-4} (mean; 95% CI: 1.57×10^{-6}–6.90×10^{-4}) CFU/28-g (mean) 70–90% dark chocolate tablet of *Salmonella* are estimated, respectively (Supplementary Table 5). When a 6 log reduction is obtained, 19 (mean; 95% CI: 0.19–86) CFU/28-ton (mean) cocoa liquor and 1.49×10^{-5} (mean; 95% CI: 1.57×10^{-7}–6.90×10^{-5}) CFU/28-g (mean) 70–90% dark chocolate tablet of *Salmonella* are estimated, respec-

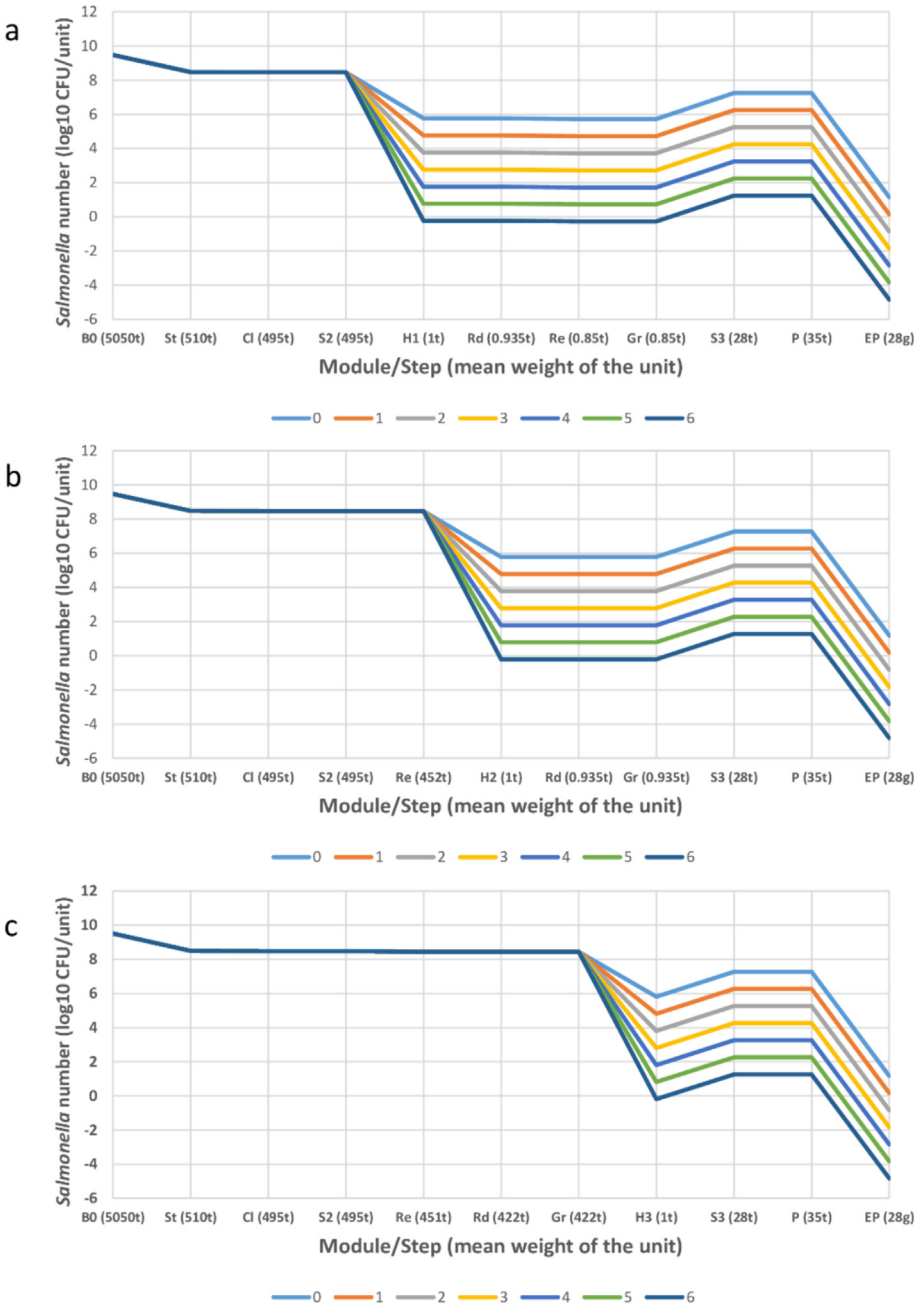

Figure 3. Estimated mean *Salmonella* numbers in each module/step of process for 0-, 1-, 2-, 3-, 4-, 5-, 6-log reduction treatment levels, (a) bean heat process, (b) nib heat process, (c) liquor heat process. The module code given in *x* axis is also described in Table 2: B0, raw whole beans; St, storage in the warehouse + transport to the factory; Cl, Precleaning (remove foreign bodies); S2, storage in the silo; H1, H2, and H3 are where the thermal treatment as CCP is applied for 3 different processes; Rd, roasting/drying; Re, breaking and winnowing into nibs; Gr, grinding into liquor; S3, Storage liquor in tank; P, further processes + mixing with other ingredients; EP, final product (70–90% dark chocolate tablet). The number after the module code represents the mean unit size (t = tons, g = gram).

tively (Supplementary Table 5). When no thermal treatment is applied, a mean of 1.85×10^7 (95% CI: 1.93×10^5–8.55×10^7) CFU of *Salmonella* is expected in 28-ton (mean) cocoa liquor, and a mean of 14.9 (95% CI: 0.157–69) CFU of *Salmonella* are expected in the hypothetical 28-g (mean) 70–90% dark chocolate tablet made from this contaminated cocoa liquor (Supplementary Table 5).

Estimated salmonellosis risk for consumption of the hypothetical dark chocolate tablet

Supplementary Table 6 and Supplementary Table 7 represent the risk characterization results showing risk per serving and annual risk per consumer respectively, when 0 to 6 log reductions are simulated. As the log reduction increases from 1 to 6 log reductions, the risk per serving and annual risk per consumer decrease by ca. 10^{-1} times for each 1 log reduction.

When a 4, 5, 6 log reduction is applied, the estimated mean risk per serving decreases to 3.33×10^{-7} (95% CI: 3.27×10^{-9}–1.59×10^{-6}), 3.35×10^{-8} (95% CI: 3.27×10^{-10}–1.59×10^{-7}), and 3.35×10^{-9} (95% CI: 3.27×10^{-11}–1.59×10^{-8}), respectively (Supplementary Table 6). When no thermal treatment is applied (0 log reduction; Supplementary Table 6), the estimated mean risk per serving is 1.60×10^{-3} (95% CI: 3.27×10^{-5}–9.39×10^{-3}). This means

that if no thermal treatment is given in the cocoa processing, a mean of 1.60 salmonellosis cases per 1000 servings is expected due to the consumption of dark chocolate. Assuming the Belgian consumer eats dark chocolate every day as the worst case (Section '**Risk characterization**'), the estimated annual risk per consumer increases obviously ca. 365 times compared to the estimated risk per serving as shown in Supplementary Table 8. As an example, if a 4, 5, 6 log reduction is achieved, ca. 12, 1 and <1 mean salmonellosis cases per 100,000 Belgian consumers are estimated in a year, respectively (Supplementary Table 7), assuming that the risk from every single exposure is independent of every other exposure. If no thermal treatment is applied, a mean of 5.86×10^{-1} annual risk per consumer represents that 58,600 salmonellosis cases per 100,000 Belgian consumers are expected in a year as the worst case (Supplementary Table 7).

Estimated salmonellosis cases for consumption of dark chocolate in the Belgian population per year

Supplementary Table 8 represents the risk characterization results showing estimated Belgian human salmonellosis cases per year due to the consumption of hypothetical end product of 70–90% dark chocolate, when 0–6 log reductions are applied. The estimated number of salmonellosis cases in the Belgian population per year decreases roughly ca. 10 times as the log reduction is increased by 1 log (Supplementary Table 8). Importantly, these estimated numbers assume that 34–87% of Belgian people are eating 18–40 g of a hypothetical 70–90% dark chocolate tablet (made from the cocoa liquor) every day in a year. Once a 4, 5, or 6 log reduction is applied in the production process, the estimated mean of Belgian salmonellosis cases decreases to 871 (95% CI: 9–4180), 88 (95% CI: <1–418), or 9 (95% CI: <1–42) per year, respectively. It is worth noting that if no thermal treatment is given in the production process, 4.26 million (mean; 95% CI: 8.53×10^{4}–2.50×10^{7}) salmonellosis cases are estimated in the Belgian population per year (Supplementary Table 8).

Sensitivity analysis

Spearman rank correlation coefficients show that only the input distribution parameter β in the dose–response model has a negative correlation with the output of risk per serving, the other 8 input distributions all positively impact the model output (Fig. 4). Notably, the initial *Salmonella* concentration in the contaminated raw cocoa beans has the greatest impact (correlation coefficient = 0.98) on the model output of risk per serving, for a 5 log reduction in the bean heat process (Fig. 4). Although only an example of the sensitivity analysis is presented, for both nib and liquor heat processes, the initial *Salmonella* concentration in the contaminated raw cocoa beans is also the factor with the greatest impact on the model output (data not shown). It is also worth noting that 'unit size_tablet' representing the serving sizes of 70–90% dark chocolate daily consumed by Belgian consumers and 'prevalence' representing the prevalence (%) of contaminated servings both impact the estimates of risk per serving, with a correlation coefficient of 0.10 and 0.08, respectively (Fig. 4).

Discussion

To obtain a dataset that can represent the microbiological variability, a 9-month prevalence study including 870 cocoa bean samples, considering the major bean origins, and production period (main or mid crops), was established by three cocoa processors (details refer to Section '**Sampling design**'). The stratification based on ca. 90% biggest country origin and 80% main crop season are representative for the total cocoa supply to Belgium or Europe. Therefore, the 870 samples are covering the expected variability. A prevalence of 8.0% *Salmonella* contaminated raw cocoa beans (after fermentation and drying in the farm) imported to Europe was found in this study. Up to now, only one study reported that *Salmonella* was detected in one out of 119 cocoa bean samples in Brazil (da Silva do Nascimento et al., 2010). However, in the 119 samples, only 29 samples were stored dried fermented cocoa beans, the other samples were cocoa beans either before fermentation, during fermentation, or during dry-

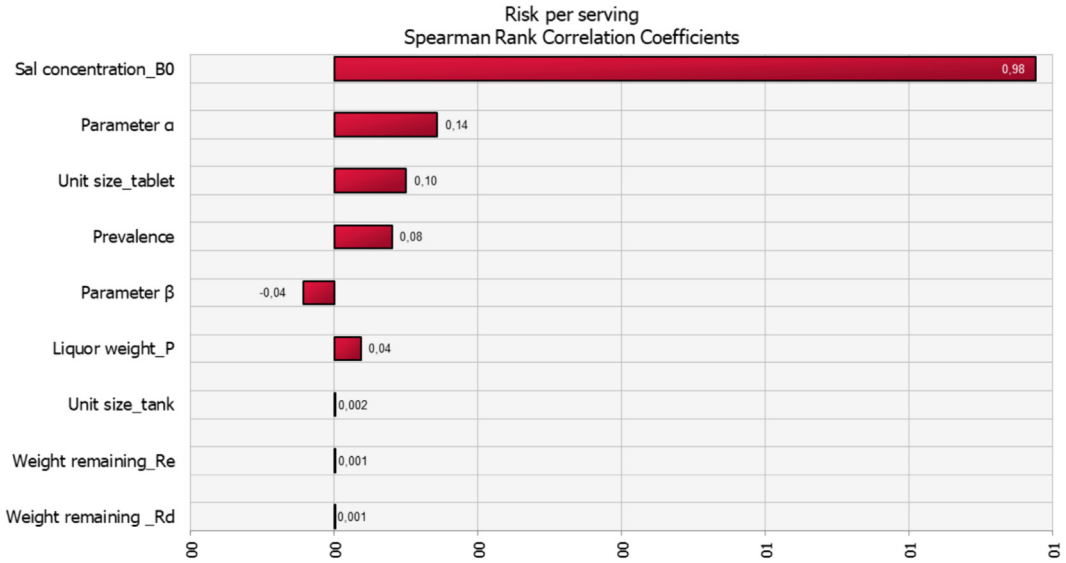

Figure 4. Spearman rank correlation coefficient for the baseline risk assessment model with risk per serving from the consumption of dark chocolate as the output variable, giving a 5-log reduction from the bean heat process as an example (the same rank for 0 to 6-log reduction). A correlation coefficient ranging from 0 to 1 means a positive correlation between the input and output, a correlation coefficient ranging from −1 to 0 means a negative correlation between the input and output. Sal concentration_B0, the initial *Salmonella* concentration in contaminated raw cocoa beans; Parameter α and parameter β represent the parameters in dose–response model; Unit size_tablet, serving sizes of dark chocolate daily consumed by Belgian consumers; Prevalence, the prevalence (%) of contaminated servings; Liquor weight_P, the percentage of liquor mixed with other ingredients in the further processes to obtain dark chocolate; Unit size_tank, the unit size of liquor tank for storage; Weight remaining_Re, the percentage of weight remaining after the beans were broken and winnowed into nibs; Weight remaining_Rd, the percentage of weight remaining after roasting and drying.

ing. Thus, the prevalence of *Salmonella* in stored dried fermented cocoa beans − which was recalled in the present study as 'raw cocoa beans' − reported by da Silva do Nascimento et al. (2010) was ca. 3% (1/29). Those cocoa bean samples were collected from three different cocoa farms in the state of Bahia, Brazil. It is also worth noting that only 25 g intact (no broken or fragmented) cocoa beans were preenriched in BPW, subsequently were enriched in Rappaport–Vassiliadis and tetrathionate broth and plated onto three different *Salmonella* selective agars (da Silva do Nascimento et al., 2010). The difference in subsample weight (25 g in the Brazil study versus 375 g in this study as mentioned in Section '*Survey of Salmonella in raw cocoa beans imported to Europe*') along with the different sample preparation (intact or fragmented/broken cocoa beans) and difference in preenrichment approach (the use of BPW or milk with supplements) will impact on the *Salmonella* detection during a survey.

From the prevalence study, a single highly contaminated sample (ca. 4 log CFU/g *Salmonella*) was detected among the 870 samples, whereas in the other 70 samples showing *Salmonella* positive detection, the pathogen was present in lower concentrations (0.008–46 MPN/g). Because of manual harvest and handling, spontaneous fermentation and drying under the sun in tropical areas, occasional *Salmonella* contamination to the cocoa beans may occur (De Vuyst & Weckx, 2016; Oyedeji et al., 2023; Ozturk & Young, 2017; Thompson et al., 2014; Winkler, 2023). *Salmonella* detected in the sample with a count of ca. 4 log CFU/g was sublethal injured, as colonies could only be noted using XLD top-layered TSA but could not detected using plating on either XLD agar (without TSA top-layer) or RAPID'*Salmonella* Agar Plates. This finding highlighted the importance of using appropriate detection methods for recovery of *Salmonella* in raw cocoa beans as they are indeed expected to be sublethal injured due to acidified pH at the end of spontaneous fermentation. It has been reported that when *Salmonella* inoculation (ca. 4 log CFU/g) was performed at the end of the fermentation, no *Salmonella* reduction was found after bean drying and storage (Nascimento et al., 2013). In conclusion, the prevalence study was important indeed to have a realistic view of the status of the 'input' (raw material contamination) for the QMRA simulation model of cocoa liquor processing and chocolate production.

The purpose of this study was to use QMRA as a tool to evaluate the log reduction in the cocoa processing, and thus, no other sources of contamination were considered in the QMRA model, such as ingredients or environmental contamination. In this QMRA, as expected, the initial *Salmonella* concentration in raw cocoa beans significantly impacts the risk per serving of salmonellosis as shown in Fig. 4. Another important factor that can affect the output (e.g. risk per serving) of a QMRA is the dose–response model. This dose–response model was conducted based on 33 reported outbreak data including strains from 7 *Salmonella* serotypes (*S*. Typhimurium, *S*. Heidelberg, *S*. Cubana, *S*. Infantis, *S*. Newport, *S*. Enteritidis, and *S*. Oranienburg) as the causative agents and outbreaks were related to food (meat, eggs, dairy products, and others), water, and a medical dye capsule (FAO/WHO, 2002). Thus, the applied FAO/WHO model is not based on information from chocolate outbreaks, for which it is known that low doses can cause disease (response). On the other hand, the *Salmonella* serotypes that can be found in tested raw cocoa beans might have different pathogenicity (and thus attack rates). The dose–response model published by Teunis (2022) conducted different models for each *Salmonella* serotype using either outbreak data or human challenge study data. However, as the present study did not aim for estimating the burden of disease caused by a specific serotype, a diversity of *Salmonella* serotypes is expected to be present in raw cocoa beans. It was considered to be more appropriate to use the generic model from FAO/WHO in the present study instead of the serotype − specific model by Teunis (2022).

Overall, the presented QMRA model provides the impact of cocoa liquor processing (0–6 log reductions) on the estimated residual *Salmonella* risk (at the population level) by means of the conversion of these cocoa liquor volumes produced from the raw cocoa beans baseline distribution into hypothetical dark chocolate tablets (with no consideration of further *Salmonella* contamination opportunities) that are set to the market for consumption.

ICMSF (2011) historically considered a minimum 6 log reduction in the heating step, but NCACC (2011) recommends a 4–5 log reduction. To determine which *Salmonella* reduction level during thermal treatment (CCP in the cocoa processing) is minimally required in the framework of a reasonable degree of protection (DOP) for salmonellosis, the approach and outcome of some other relevant QMRA studies were compared with the present QMRA study. There was only one other QMRA study published on *Salmonella* in chocolate. However, an unfortunate mistake (wrong parameter α and β position) in the dose–response formula used in the QMRA of Campagnollo et al. (2020) might have been the cause for ending up in a generally much higher risk than expected. Another QMRA study that was of interest to compare with the present study was the assessment of the risk of salmonellosis arising from the consumption of almonds or pecans in the United States by Farakos, Pouillot, Johnson, Spungen, Son, Anderson, Davidson, et al. (2017) and Farakos, Pouillot, Johnson, Spungen, Son, Anderson, and Van Doren (2017), as it also evaluated the log reduction established by heat processing and estimated residual risk. These QMRA studies indicated that a minimum 4 log reduction of *Salmonella* was required and considered sufficient during the thermal treatment process for almond and pecan kernels, resulting in the estimated mean salmonellosis cases below one case per year in the United States. However, even when a 6 log reduction was applied in the presented QMRA model for cocoa liquor processing, the estimated mean salmonellosis cases in Belgium attributed to eating the hypothetical derived dark chocolate tablets were 9 (95% CI: $<$1–42). A further log reduction (i.e. $>$6) could be difficult to achieve in the cocoa liquor single-step thermal treatment considering the desired cocoa flavor and color. Furthermore, it should be taken into account that the QMRA model did not consider any possible *Salmonella* reduction from other processes that might generate heat inactivation (e.g. grinding or conching).

However, as it was shown that the 'input' drives the outcome risk estimates (Fig. 4), it is only fair to also compare the 'baseline' risk if no thermal treatment is applied. When no thermal treatment (0 log reduction) would be applied, the estimated mean risk per serving (serving size of almond, 26.3 ± 30.1 g; serving size of pecan, ca. 1–120 g) was 9.3×10^{-7} and 1.3×10^{-6} for almonds and pecans, the latter thus consumed as a core product (product with $>$80% content of almonds or pecans) without cooking at home (Farakos, Pouillot, Johnson, Spungen, Son, Anderson, & Van Doren, 2017; Farakos, Pouillot, Johnson, Spungen, Son, Anderson, Davidson, et al., 2017). Compared to the present QMRA starting from cocoa beans to chocolate, if no thermal treatment would be applied during the cocoa liquor processing (assumption of 0 log reduction), the estimated mean risk per serving was 1.6×10^{-3} (Supplementary Table 6). This is because the initial baseline distributions of *Salmonella* concentrations in contaminated raw almonds (mean: −4.97 log CFU/g) and pecans (mean: −5.72 log CFU/g) estimated by Farakos et al. (2017a, 2017b) are overall situated at lower levels than in raw cocoa beans (mean: −0.87 log CFU/g) estimated in the present QMRA based on the survey results in the present study. Besides, the prevalence of *Salmonella* in raw almonds (ca. 1%) and pecans (ca. 1–3%) are also found to be lower than in raw cocoa beans (ca. 8%). The lower prevalence and contamination levels of *Salmonella* in pecan and almond nuts might be because pecan and almond kernels are better protected by their shells than cocoa beans, and no spontaneous fermentation is involved in the production process of pecan and almond nuts.

The U.S. EPA considered one illness (or infection) per 10,000 individuals in a given year, which is $\leq 10^{-4}$ per person per year (pppy), as a reasonable guideline for drinking water regarding the waterborne

Table 4
Distribution of estimated Belgian human salmonellosis cases per person per year from consumption of a hypothetical dark chocolate tablet and a worst-case daily consumption to evaluate impact of 7 different log reduction treatment levels.

Log reduction	Mean	SD	Quantiles of variability		
			2.5%	50%	97.5%
0	3.64E−01	6.58E−01	7.43E−03	1.56E−01	2.13E+00
1	5.36E−02	1.90E−01	7.44E−04	1.61E−02	3.30E−01
2	6.49E−03	4.69E−02	7.45E−05	1.62E−03	3.51E−02
3	7.11E−04	9.42E−03	7.45E−06	1.62E−04	3.54E−03
4	7.31E−05	1.21E−03	7.45E−07	1.62E−05	3.54E−04
5	7.33E−06	1.25E−04	7.45E−08	1.62E−06	3.54E−05
6	7.34E−07	1.26E−05	7.45E−09	1.62E−07	3.54E−06

Belgian cases per person per year = Belgian cases per year/Belgian population.
SD, standard deviation.

disease burden (U.S. EPA, 1989). This degree of protection (DOP) was considered as a benchmark that could be used in this QMRA. The estimated mean of Belgian human salmonellosis cases pppy due to the consumption of dark chocolate for the treatment level from 4 to 6 log reduction are 7.45×10^{-5}, 7.45×10^{-6}, and 7.45×10^{-7}, which are lower than 10^{-4} pppy (Table 4). However, the 97.5% upper bound for 4 log reduction treatment is 3.58×10^{-4} (Table 4), which is higher than 10^{-4} pppy. Therefore, DOP with a minimum 5 log reduction can be considered acceptable, considering the variability (95% CI). A 5 or 6 log reduction was expected to result in a calculated mean of 75 salmonellosis cases out of 10 million (7.48×10^{-6} pppy in Table 4), or 8 salmonellosis cases out of 10 million (7.49×10^{-7} pppy in Table 4) Belgian people per year, respectively. The estimated risk is calculated without considering the additional thermal steps where *Salmonella* reduction takes place and is part of the conventional cocoa bean processing (e.g. grinding). The risk estimated from this QMRA is limited to salmonellosis caused by the consumption of 70–90% dark chocolate tablets specifically in Belgium and can be extended to other EU countries by replacing the consumption and inhabitant data. With more available data fitting into the data gaps in the future, this QMRA could be improved to reduce uncertainties.

The outcome of the residual salmonellosis risk for the given target levels (minimum 5 log reduction) for thermal treatment depends upon the initial prevalence and contamination level of the incoming raw product (the fermented dried cocoa beans). The baseline of *Salmonella* prevalence has been determined in the context of the given current practices in good farming and postharvest practices for cocoa production and needs to be further maintained and continuously invested in (Levai et al., 2015). Also in chocolate manufacturing, it is important to prevent the entry, spread, and growth of *Salmonella* in the production environment, by having appropriate prerequisites like zoning, GMP, pest control in place (Bourdichon et al., 2021; Chen et al., 2009a, 2009b; Podolak et al., 2010; Scott et al., 2009). Environmental sampling, as a means of verification of zoning measures in place, has a long historical track record in the chocolate industry of being very effective in the search for *Salmonella* in the production environment. It is clear that to achieve a desired DOP, the overall food safety management principles and the manufacturer's adherence to properly validated and implemented HACCP plan for cocoa (liquor) processing and chocolate production are necessary. Furthermore, when other ingredients are used in chocolate making, a dedicated supplier selection and maintenance program is needed to prevent recontamination of the (dark) chocolate. Lastly, verification of process controls by sampling and testing of end products is a commonly applied quality control activity in the chocolate industry (U.S. FDA, 2022; Zwietering et al., 2016). In addition, there is a track record of evidence that in outbreaks, only rarely the 'raw cocoa beans' were the source of *Salmonella* (Supplementary Table 1). This confirms the adequacy of the current practices ensuring/aiming at a minimum 5 log reduction at single-step thermal treatment in cocoa processing. In summary, as zero risk does not exist, the risk of salmonellosis should be kept as low as reasonably achievable and for that combined preventive measures are needed to cover the whole chain from farm to fork.

CRediT authorship contribution statement

Xingchen Zhao: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Liesbeth Jacxsens:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Vesela Tzeneva:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Michiel Kokken:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Anett Winkler:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Cécile Vadier:** Conceptualization, Methodology, Resources, Writing – review & editing. **Nicolau de Toledo:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Tomasz Seliwiorstow:** Conceptualization, Funding acquisition, Investigation, Resources, Writing – review & editing. **Mieke Uyttendaele:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jfp.2024.100327.

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