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Implementation of omics tools for infant food microbial safety

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ABSTRACT

Microbial safety of infant food is a priority for food producers, regulatory authorities and consumers. Infant food is destined to a particularly sensitive section of the population that is more susceptible to foodborne diseases if compared to the general population. Preventive approaches are applied during production and commercialization that rely on the principles of Hazard Analysis Critical Control Points and on Good Hygiene Practices to guarantee food safety. Nevertheless, microbial safety hazards in infant foods still pose a public health risk. With the purpose of better understanding why and how pathogenic microorganisms contaminate infant food, an approach that integrates omics methods is here proposed and reviewed. Omics approaches are employed to study the microbial ecology of foods, to investigate the interactions of microorganisms within an ecosystem and to delineate the behavior of microorganisms during food processing.

1. Introduction

Food industry and regulatory authorities strive to provide safe foods to the consumer. When infant food is concerned, there is an inherent increased concern and care in all the steps involved in the production, given the nature and sensitivity of the final consumer. With the term infant food, a diverse group of food commodities is intended, and such diversity has increased in recent years to respond to the desire of consumers in terms of nutritional characteristics, convenience of use, environmental sustainability, ethical aspects of the products. However, the core of infant food is composed of *Infant formulae* and *follow on formulae* and is intended for children below the age of 12 months. The distinction between the two is that infant formulae serve as sole source of nutrition or integrate breast feeding in infants, while follow on formulae are designed and intended to be used during the weaning period, in combination with other foods. A common characteristic of these two types of foods is that they are dehydrated. This characteristic implicates that the products cannot be sterilized and therefore low levels of microorganisms are potentially present. When pathogenic microorganisms are present, a foodborne disease may develop following ingestion of the contaminated product. In fact, infant formulae and to a lesser extend follow-on formulae have been implicated in outbreaks of foodborne disease in infants. Therefore, the microbiological safety of infant food and specifically powdered formulae is of outmost importance to reduce the risk to public health. This review aims at discussing the current status regarding microbial safety of infant food, with particular emphasis, on powdered infant food, and at presenting the approach employed within the SAFFI EU-China project that has as a purpose to further enhance the already high level of safety of infant foods providing knowledge and tools to both industry and regulatory authorities.

2. Characteristics of powdered formulae

The microbiological stability at room temperature, of infant food powders (most commonly milk-based and cereal-based), relies on the low water activity (a_w).[1,2] In fact, in such products a_w is usually in the range of 0.3-0.6 and in any case, it is below 0.8-0.82 that is considered the lowest limit permissive for growth of foodborne pathogens. However, the products are not sterile and may contain low concentrations of microorganisms.[3] The characteristics of the powdered formulae do not allow a processing step that could eliminate/reduce the microbial load of the final product or remove potentially pathogenic microorganisms. Indeed, powdered infant formulae have been involved in sporadic cases or outbreaks of foodborne disease, following ingestion of a contaminated product. Epidemiological investigations have highlighted the importance of the procedures of reconstitution of the powdered formula (by water addition and concomitant increase of the a_w) and subsequently, the conditions (temperature, time) of storage of the ready to

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use, reconstituted product. Water addition leads to a_w conditions favorable for growth of microorganisms. If the product is not immediately consumed or is not stored at refrigeration temperature, then cell proliferation occurs leading to increase in the microbial load. Ingestion of such product represents a high risk for disease development in the consumer.

The infant food formula production process involves some common steps that are: mixing of ingredients (that may have been subjected previously to a microbiocidal treatment), homogenization, drying, packaging. Two types of procedure may be employed for the production: the wet and the dry method. In the wet procedure, the mixing and homogenization take place first, followed by drying and packaging. In the dry procedure, the ingredients are first dried and then mixing and packaging take place. Microorganisms that can be detected in the final product may originate either in the ingredients employed or in the plant production environment. Contamination from the environment needs to be reduced to the minimum since there will be no microbiocidal treatment prior to the packaging and consumption of the product. Thus, Good Hygiene Practices (GHP) during manufacturing become indispensable to guarantee the microbiological safety of the final product. Furthermore, GHP during preparation and use of powdered formulae are equally important, particularly in health care facilities where large quantities of infant food may need to be prepared and possibly stored to satisfy the various needs of neonates (Fig. 1).[4]

3. Pathogens of concern in powdered infant formulae

The large scale of production of infant formula and follow-on formulae, both distributed worldwide, and the relatively low number of infections in infants indicates that the products are normally safe.[5] Nonetheless, issues relating to enteric and foodborne diseases are of particular relevance to paediatricians because the age-specific incidence rates for many of the most commonly reported enteric and foodborne pathogens are highest among infants and young children.[6] Sporadic cases or foodborne outbreaks due to consumption of contaminated infant formula are occasionally reported worldwide.[3] Clear evidence of causality links two pathogens, namely *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*) and *Salmonella enterica* with illness in infants. Infant formula, contaminated with these two microorganisms has been proven epidemiologically and microbiologically to be responsible for infection in infants.

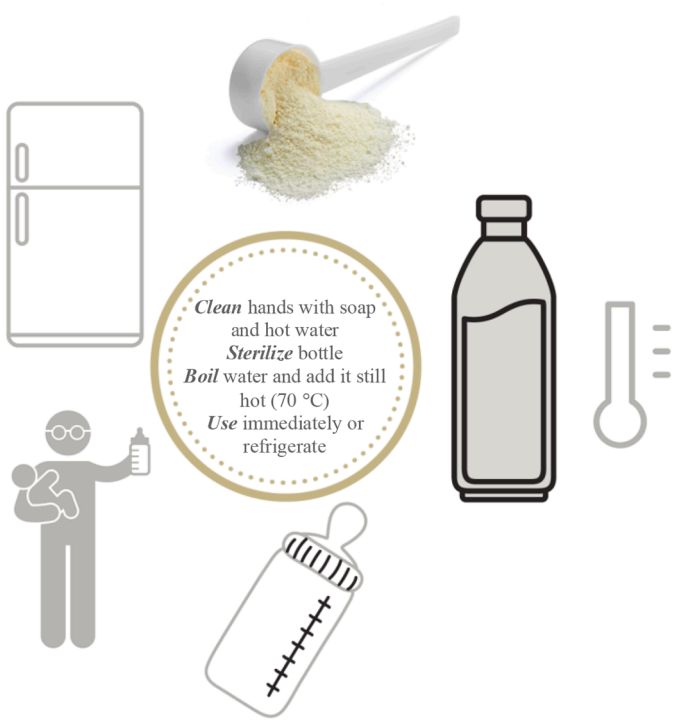

Fig. 1. Basic good hygiene practices for the preparation of infant food from powdered formula (for more information see[4]).

Infections by *C. sakazakii* mainly concern neonates, pre-term, underweight or immunocompromised infants and symptoms range from severe diarrhea to systemic infections, necrotizing enterocolitis, sepsis, meningitis. The mortality rate may be as high as 50% or may lead to serious, long term neurological complications (sequelae) (cdc.gov./cronobacter/technical.html). The habitat of *C. sakazakii* has not yet been identified. It has been consistently reported that contamination takes place from the environment, within the food production plant. For this reason, particular emphasis is placed in the prevention of such contamination through good manufacturing practices and good hygiene practices.

S. enterica is a well- recognized foodborne pathogen, affecting consumers of all ages and traditionally associated with food products of animal origin. In recent years, it is increasingly being connected to foodborne outbreaks due to consumption of foods of plant origin. Several outbreaks of salmonellosis have been traced to dried milk products and research has shown that failures in the production or presence of *Salmonella* in zones that are difficult to maintain clean were responsible for the contamination.[5] In infants, salmonellosis is manifested as gastroenteritis, but when it is invasive (extra-intestinal) it may lead to serious complications including bacteremia, arthritis, osteomyelitis, fatal meningitis.[7,8]

Other pathogenic microorganisms that may contaminate powder infant formula and are known to cause disease to humans mainly belong to the family *Enterobacteriaceae*. However, in contrast to the two pathogens described above, the epidemiological and microbiological evidences do not support a clear connection between consumption of infant food and infection caused by members of the genera *Klebsiella, Citrobacter, Hafnia*.[3] Similarly, for sporeformers *Bacillus cereus, Clostridium perfringens* and *Clostridium botulinum* no causal relation has been identified. *B. cereus* is a known enteropathogen that causes intoxication, with emesis as the most common symptom or infection, with diarrhea as most common symptom. Intoxication and/or infection disease due to *B. cereus* usually develop sporadically and do not cause outbreaks. Low level contamination of infant formulae is to be expected, mainly due to the heat resistant endospores that persist in the final product.[9,10] *Staphylococcus aureus* and *Listeria monocytogenes* are two additional foodborne pathogens of concern that may cause intoxication and infection respectively in infants but are not considered common contaminants of powdered infant formulae. Nevertheless, *L. monocytogenes* is an ubiquitous microorganism that may potentially contaminate a diverse array of raw materials. In addition, both *L. monocytogenes* and *S. aureus* may be involved in secondary contamination of food products.[11]

4. Food safety management systems and microbiological criteria

In order to satisfy the high food safety standards requested by modern consumers and importantly to protect consumer health from hazards that may be transmitted with food, the food industry employs science-based, preventive approaches to produce and commercialize foodstuffs. The food industry today cannot rely on end-product testing to guarantee food safety. End-product testing is impracticable, considering the amounts of foods produced and the extend of globalization of the food market. More importantly, it is inefficient in identifying food safety breaches that could occur during food production. Therefore, the food industry and competent authorities/regulatory bodies have adopted a risk-based approach to food safety that essentially follows the principles of Hazard Analysis Critical Control Points (HACCP). Hazards (chemical, biological and physical) that may potentially occur during food production (at any stage of the food chain) are identified and appropriate control measures are put into place to limit or prevent occurrence of the

hazard. In parallel, procedures are foreseen that are intended to verify that the HACCP system is working effectively and that the potential hazards are controlled, resulting in a safe product.[12] Intrinsically intertwined with the HACCP system are the Good Hygiene Practices (GHP). These are all the fundamental measures and conditions applied at any step within the food chain to provide safe and suitable food.[13] Microbiological analysis of foodstuffs to verify the efficacy of all the procedures aiming at producing safe foods is advisable. In this context, microbiological criteria that are either used to distinguish safe/unsafe foods or are used to highlight the correct/problematic functioning of the production process have been defined. For foods prepared and commercialized in Europe, EU legislation 1441/2007 defines such criteria (Table 1).

5. Bias of traditional microbiological analysis – bias of cultivation step

Traditional microbiological analyses that rely on the use of culture media have been fundamental in detecting and studying microorganisms in foods. Nevertheless, they present inherent limitations (Fig. 2). Such analytical approach is culture-dependent and by default, may only reveal the presence of microorganisms that at the moment of analysis have the ability to proliferate in synthetic microbiological media. However, under certain conditions, usually conditions of stress, microbial cells may be alive but not able to proliferate. This condition is termed Viable Not Culturable State (VBNC) and it is possible that during food production, microorganisms encounter stressful conditions that prompt the entrance in the VBNC state.[16,17] Also, the composition of the medium, and overall the growth conditions, need to be permissive to growth for a given microorganism. If the conditions are not permissive to growth (for example when an essential nutrient is missing) for a specific microbial group, then the result of the analysis will be a false negative (the microorganism is present in the sample, but is not detected by the method employed). Further, it should be mentioned that when various microbial groups are present in the sample at concentrations that diverge, only the most abundant populations will be detected unless appropriate selective conditions are imposed during the culturing procedure. Evidently, the culture-dependent detection of microorganisms is biased and may result in a distorted view of the microbial ecology of a given sample being analyzed.[18,19]

When performing a microbiological analysis, the aim is to detect (and/or enumerate) one or more of the microbial groups that are present in a sample. Oftentimes, microbiologists are also interested in expanding their objectives and they pursue a more detailed description of the microbial ecology of the sample by isolating microorganisms and studying them in greater detail to understand their role in a specific ecosystem. This is traditionally performed using pure cultures of microorganisms. More specifically, a microorganism, isolated from the rest of the microbial community, is subjected to various tests with the purpose of obtaining detailed information regarding its behavior. The ability to utilize different substrates (carbon and nitrogen sources), the velocity of growth at different pH, a_w or temperature conditions, the resistance to antimicrobial compounds, tolerance to growth limiting substances are some examples of physiological tests that are helpful in understanding the behavior of a microorganism. This information is then extrapolated to predict how the same microorganism would behave in a real food. There is however a fundamental difference between the experiments performed *in vitro* and the actual food. The food (at any stage of the food

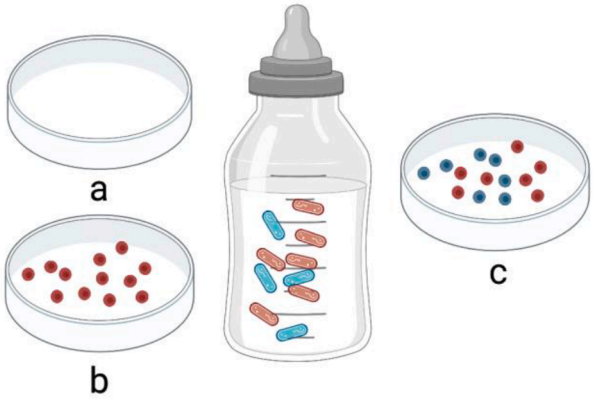

Figure 2. Possible outcomes of a microbiological analysis, based on cultivation, of a contaminated sample. a. No microorganism detected; (i) the target microorganism was present in the sample in a VBNC state, (ii) prior knowledge regarding the contamination was not informative and the media used not appropriate for the detection of the contaminating microorganisms. b. Detection of part of the microbiota contaminating the sample; a dominant microbial population conceals minor populations. In both cases, the potential safety risk for the consumer is underestimated. c. The result of the analysis reflects the actual contamination of the sample.

Table 1
Microbiological criteria in powdered formulae (based on EU regulation 2073/2005, 1441/2007[14,15])

Food Safety Criteria; they define the acceptability of a food product or a batch and they apply for products on the market. Food business operators (FBO) have to comply with them and the testing for these criteria can be used for the verification of the HACCP and GHP procedures. Competent authorities may also sample and test for these criteria in the context of verification of compliance of food business operators.

Food category	Microorganism	n (number of sampling units to be analyzed)	c (number of units that may overcome the limit)	m (limit)	Actions
Dried follow-on formulae	*Salmonella*	30	0	Absence in 25 g	In case of unsatisfactory results, the food product is removed/recalled from the market and the FBO should investigate the reasons that led to the unsatisfactory result, eventually modifying the food safety procedures
Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age	*Cronobacter sakazakii*	30	0	Absence in 10 g	

Process Hygiene Criteria; they indicate the acceptable functioning of the production process and they do not apply to products on the market. Food business operators use such criteria to monitor the production process and employ corrective actions if unsatisfactory results are obtained.

Food category	Microorganism	n (number of sampling units to be analyzed)	c (number of units that may overcome the limit)	m (limit)	Actions
Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age	*Enterobacteriaceae*	10	0	Absence in 10 g	In case of unsatisfactory results, corrective actions need to be taken, including improvement in production hygiene to minimize contamination
Dried follow-on formulae	*Enterobacteriaceae*	5	0	Absence in 10 g	

chain) is a complex ecosystem in which biotic and abiotic factors influence each member of the microbial community. Importantly, in the food, the different microbial groups interact in various ways and such interactions eventually affect the population dynamics and the survival or disappearance during food processing. Interactions among microbial groups and with the surrounding environment (i.e. the food or the processing plant) cannot be captured when performing experiments *in vitro* and in pure culture.[20]

Foodborne pathogens, if present in a food usually consist in a minor population of the entire microbiota. For this reason, enrichment approaches are necessarily employed. Through enrichment, an effort is made to increase the concentration of the target microorganism. Usually, this is achieved by using selective agents that should inhibit the competing microbiota. Such approaches are known to differentially influence microorganisms and therefore the outcome of the analysis may be altered when compared to the actual situation in the food at the moment of sampling.[20]

6. Contribution of omics in food microbiology

Already in the late 90's the limitations described above had been recognized through the application of analytical approaches that bypass the cultivation step and rely on detection of genetic material that can be robustly associated with the presence of a given microorganism in a sample. These approaches are collectively termed culture-independent approaches and have been instrumental in the detailed study of the microbial ecology of foods, with particular emphasis on fermented foods.[21] Fingerprinting techniques, particularly the Denaturing Gradient Gel Electrophoresis (DGGE), allowed for the first time the direct analysis of nucleic acids extracted from food samples capturing the complexity of the microbial ecology without the bias of the cultivation step.[22]

The application of DGGE in food analysis paved the way for the use of other culture-independent techniques. The evolution in the DNA sequencing technology and the introduction of Next Generation Sequencing (NGS) in microbiological analysis further improved the detection and description of microbial communities in foods. NGS is a massively parallel sequencing technology that generates millions of sequencing reads. The sequencing reads are then processed and offer taxonomic or functional information (Fig. 3).

The NGS technology may be applied to DNA originating from a food sample to study the microbial communities. In amplicon sequencing or metabarcoding (also metataxonomics), a PCR step precedes the sequencing. A genomic region that is present in all members of the community is amplified in multiple copies and then sequenced. Usually, the target to aplify is chosen based on the taxonomic information it may provide. Commonly, genes that encode for ribosomal RNA molecules are targeted. In this way, potentially all members of the community are represented (amplified) and after sequencing they can be identified (by comparison with available databases). After data analysis, the output is taxonomic information; the composition of the microbial community can be obtained. Amplicon sequencing has been extensively used to study the microbial ecology of foods, particularly the evolution of microbial populations during production of fermented foods but also to explore the microbial spoilage phenomena.[23]

By metagenomics or shot gun sequencing it is possible to obtain information regarding the genomic content of the different members of the microbial community. Therefore, potential functions are predicted.[24] In this case, the DNA extracted from a sample is directly subjected to sequencing. Metagenomics are being explored as a tool to detect and characterize pathogenic microorganisms in foods and food producing environments. Importantly, when using a metagenomic approach, it is possible to retrieve information regarding serotype, virulence genes, antimicrobial resistance genes.[24] This type of information is particularly relevant in understanding the potential risk associated with a food sample. From metagenomic sequencing data it is possible to reconstruct the whole genome of microorganisms. This permits to trace a specific

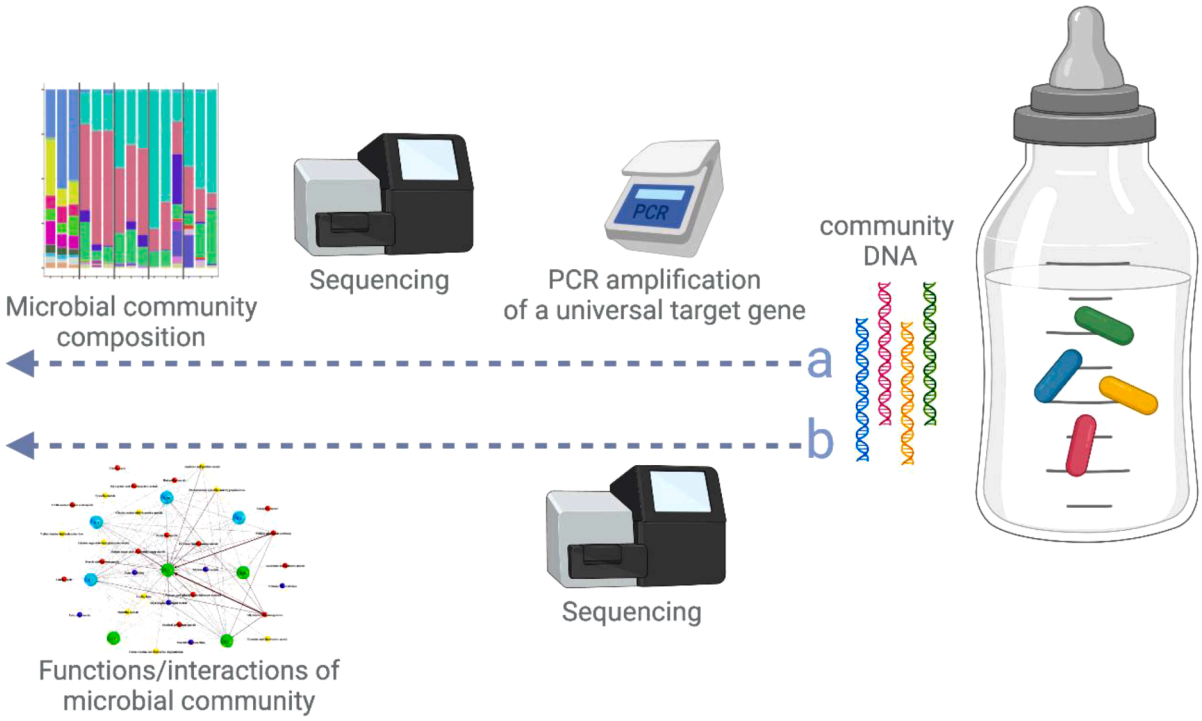

Figure 3. Next Generation Sequencing applications. *a. Amplicon sequencing* (also referred to as metabarcoding or metataxonomics); total DNA extracted from infant food sample is first subjected to Polymerase Chain Reaction to amplify a target gene, common to all members of the microbial community. The amplification product is then sequenced. The sequence information is used to deduce the composition of the microbial community. *b. Shot gun sequencing or metagenomics*; total DNA extracted from infant food sample is directly sequenced. The sequencing provides information regarding the functioning of the microbial community (for example metabolic potential, virulence potential, antimicrobial resistance). Created with BioRender.com

biotype across samples that may be related in time or space.[25] For pathogenic microorganisms this could be useful in tracing routes of contamination. Due to the low abundance of pathogenic microorganisms in foods, it may be necessary to perform a short enrichment coupled to deep sequencing. Importantly, metagenomics can be used to observe the dynamics of mixed populations that are present in the food together with the target pathogenic microorganisms during enrichment and optimize the process to maximize its efficiency.[26]

Going beyond the genetic material, it is possible to also analyze total RNA, total proteins or metabolites of a sample. Omics is a term used to encompass the analysis of macromolecules (DNA, RNA, proteins) and metabolites originating from a sample. If the sample being analyzed is a food containing mixed microbial communities, then the macromolecules and metabolites derive from all the microorganisms present in the sample. In this case the term meta-omics is employed. Analysis of molecules such as RNA, proteins and metabolites is of relevance because they provide an overview of the activity of the microorganisms in the sample. Also, they may be present at a concentration above the detection limit even when a microorganism is not prevalent in the sample. Therefore, it may be possible to detect low abundant populations targeting RNA, proteins or metabolites. By combining different omics approaches it is possible not only to detect a target pathogen but also to explore how it interacts with the rest of the microbiota and how it is influenced and behaves based on the environmental conditions that prevail. This information is fundamental since it is known that during food processing microorganisms are subjected to a changing environment and they activate mechanisms to adapt accordingly. Therefore, omics offer the opportunity to move from the determination of the presence of pathogenic microorganisms in foods towards the definition of their behavior.[27] Understanding the microbial behavior under food processing conditions will lead to a refined risk assessment for pathogenic microorganisms in foods[23,28]

In particular, the metabolomics approaches are emerging because they are able to reveal the phenotypic profile of the microbiota. At principle, metabolomics mainly targeted the most abundant metabolites. Nowadays, less abundant compounds with very high informative potential are the focus of increasing interest. Among the large diversity of microbial secondary metabolites, low molecular-weight volatile organic compounds (VOCs) have received growing attention in the past decade. Microbial VOCs (mVOCs) are typically released in a multifarious and dynamic bouquet, essentially originating from the catabolic background, and comprise a majority of low-complexity, rather lipophilic compounds.[29] The volatolome – the VOC profiling of a biological tissue or fluid – has, for instance, already shown its relevance for revealing the exposure of environment,[30] food[31,32] or human consumer[33–35] to chemical hazards.

In order to phenotype complex ecosystems, volatolomics requires the most comprehensive possible profiling of the VOCs released by the different micro-organisms at very variable levels ranging from pg/g food (ppb) to μg/g food (ppm). Today, solid phase micro-extraction (SPME) coupled with gas chromatography and mass spectrometry (GC-MS) is certainly the most frequently used technique in volatolomics. By concentrating the analytes by means of an adsorbent polymer, SPME allows rapid and automated extraction of VOCs. However, because of its limited surface of adsorption, this method suffers from competition phenomena between VOCs related to their adsorption on the polymer.[36] These competition phenomena often limit SPME use to semi-quantitative issues and raise challenges for measurement uncertainties. The latest generations of automated dynamic headspace extraction systems (DHS) might represent a first alternative option that would deserve to be benchmarked with SPME. This technique is also based on VOC trapping on a polymer. Due to its greater adsorption capacity, the implementation of DHS might limit competition phenomena compared with SPME and might then significantly improve the quantification.[37] The static headspace extraction (static HS) might also be a second alternative to SPME since it guarantees a robust VOC quantification. However, the very poor extraction yield requires coupling with the latest generation of mass spectrometers with a very high sensitivity such as the hybrid quadrupole-Orbitrap® high resolution mass spectrometers.[38] Fig. 4 presents an example of a typical workflow commonly implemented in volatolomics studies.

In view to capture the bigger picture in which the pathogens are influenced by both the food environment and the other organisms present,[18] volatolomics may provide a promising alternative to more classical metabolomics platform to reveal significant changes in the metabolism of single culture[30] or microbiota.[34] Depending on the parameters of the food processes, the food microbiome structure could be impacted, thereby creating conditions that could favor activation or inhibition of pathogen growth. A volatolomics-based strategy could be implemented to highlight the characteristics of the microbiota that may restrain or enhance persistence of pathogens. In addition to HACCP approach tracing food pathogens along the entire food chain, the detailed characterization of the food volatolome upstream or at these critical points could thus provide relevant information in order to explore pathogen behavior in samples or processing conditions that are relevant for food safety and propose predictive models to refine microbial risk assessment.

7. The SAFFI approach

The SAFFI project aims to improve infant food microbial safety providing new knowledge to the food industry and competent authorities regarding the prevalence and behavior of pathogens. Omics approaches is the fil rouge in the effort to reach this aim. Two distinct but complementary objectives are being sought.

The first objective is to perform a detailed survey of the microbiota of raw materials, intermediates, final products and importantly of the environment under real production conditions. For this purpose, an intensive sampling campaign has been implemented, covering different seasons throughout the year and focusing on collecting samples that can be correlated (in time and space). Also, relevant metadata are being collected (particularly focusing on physicochemical parameters of the samples). These samples are analyzed with optimized protocols following a traditional, culture dependent approach and a culture independent, omics-based approach. In this way, a comprehensive description of the microbiota will be obtained. The presence/absence of pathogenic microorganisms may be then correlated with particular characteristics of the microbiota in the samples, the distribution in time or space. Further, routes of contamination within the processing plant may be identified. Such type of information is critical in adopting a preventive approach that is based on knowledge and data within a particular production but may also be integrated into a refined risk assessment for infant formulae.

The second objective is to investigate the behavior of *L. monocytogenes*, chosen as a model foodborne pathogen, under *in vitro* conditions that mimic the food production process. In particular, the goal is to delineate the response of the microorganism to various types of stress conditions that are relevant to food production. Omics will be implemented for this purpose as well. Ultimately, biomarkers of adaptation or robustness may be identified that could also have a predictive character. This information will be useful in the exposure assessment step of risk assessment.[28]

8. Conclusions

Infant microbial food safety is of extreme importance. Significant interventions that aim at improving microbial food safety have been consolidated in the last 20 years. The HACCP approach, GHP, specific guidelines for producers of infant formulae have helped in making this type of products reach a high standard of safety. Nevertheless, contamination by dangerous foodborne pathogens remains a potential threat to public health and outbreaks of disease due to contaminated

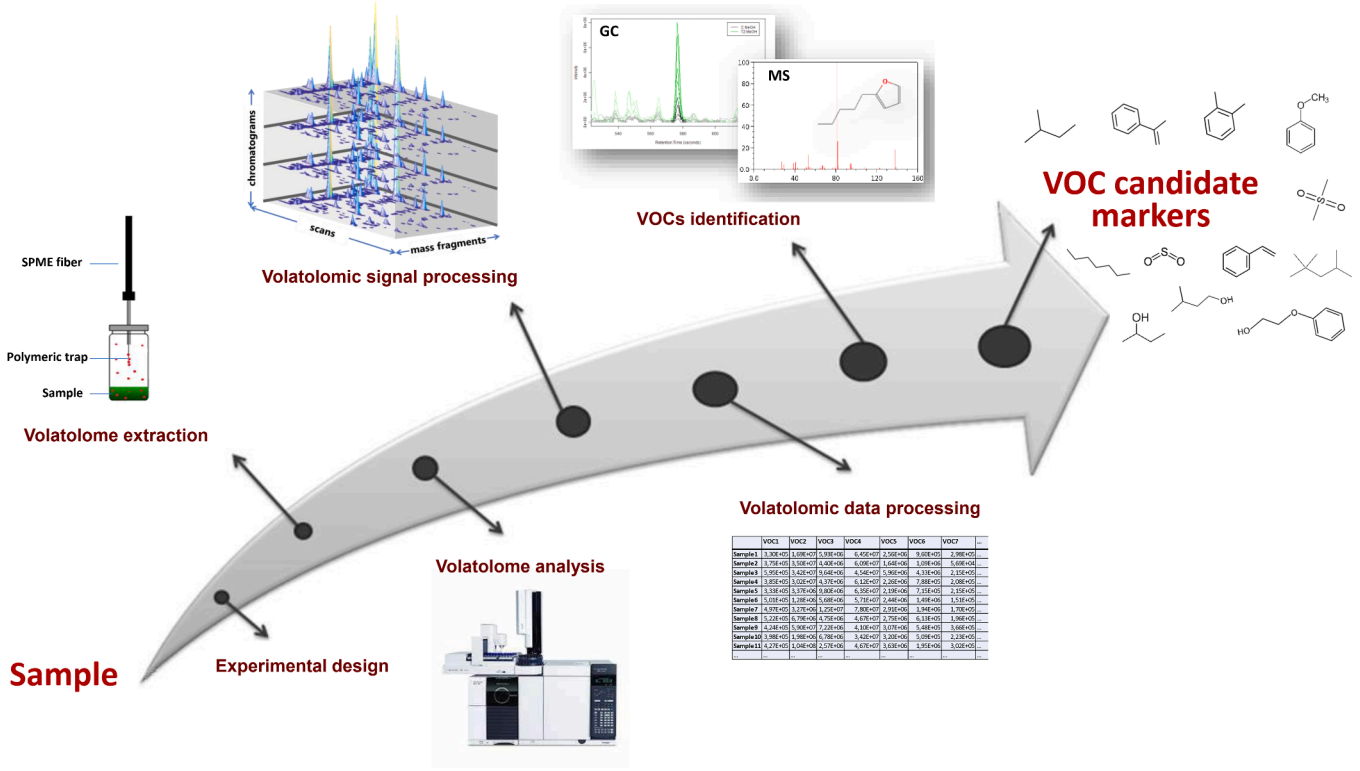

Figure 4. Typical workflow in volatolomics.

infant formulae are still occasionally reported. To tackle this safety issue, omics tools are important in identifying contamination routes, highlighting microbial interactions influencing pathogenic microorganisms, understanding their behavior under food production conditions. Generating omics data that could be integrated into risk assessment is the purpose of the SAFFI project with the overarching aim of further improving infant food microbial safety.

Declaration of Competing Interest

The authors declare no conflict of interests.

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