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Association of fungal genera from spoiled processed foods with physicochemical food properties and processing conditions

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ABSTRACT

The processing conditions and physiochemical properties used in food manufacturing create niches which support the growth of a limited number of spoilage fungi. This study was designed to evaluate the influence of intrinsic and extrinsic food product variables on the identity of spoilage fungi genera isolated from commercially produced foods. The spoilage etiology was identified in 127 products through ITS region sequencing. The prevalence and diversity of the identified spoilage fungi were evaluated in relationship to product-specific attributes using various descriptive statistics and a bipartite network analysis. Additionally, recursive partitioning was used to generate a classification tree with the outcomes, genera of the spoilage isolates, divided into increasingly homogenous subgroups. All of the isolated fungi belonged to the Ascomycete phylum, except four mucoralian isolates and the basidiomycete *Rhodotorula*. The occurrence of filamentous fungi repeatedly isolated ranged from 2% (*Phoma* spp.) to 18% (*Penicillium* spp.). In order of decreasing contribution to subgroup homogeneity, the split rules for the classification tree were based on process, water activity, food matrix category, and pH. Fungal genera representation in the terminal nodes indicated that production failures, in addition to product-specific attributes, were responsible for determination of the most probable spoilage organism.

1. Introduction

1.1. Fungal food spoilage

The select members of the fungal kingdom which are capable of contaminating and proliferating in commercially processed foods cause significant economic and security challenges for manufacturers, from shrink to consumer dissatisfaction. Approximately 25% of food waste and loss in North America is due to microbial spoilage (Gram et al., 2002), and fungi represent the most important group of spoilage microbes responsible for these losses (Sperber, 2009; Snyder and Worobo, 2018a). It is estimated that fungi are responsible for 5–10% of all food waste and loss in developing countries, comprehensive of abiotic and biotic causes (Pitt and Hocking, 2009). Filamentous fungi, commonly referred to as “molds” within the food industry, are the most common food spoilage microbe throughout the supply chain, across food sectors, and are even associated with spoilage of highly processed, high stability products (Criado et al., 2005). However, the identity of the spoilage microorganism, bacterial or fungal, responsible for the spoilage of raw ingredients or finished product depends on a combination of physical and chemical environmental factors.

Fungi are prolific food spoilage organisms. The majority of fungal species are saprobic, adapted to nutrient derivation from non-living organic matter. These fungi are chemoheterotrophic and each express a suite of extracellular enzymes that, collectively in a complex ecosystem, are capable of digesting structural organic polymers during vegetative decomposition. Fungi function similarly in food products. However, classical fungal ecology often evaluates decomposition and nutrient dynamics in complex polycultures, whereas in food processed for stability and shelf-life extension, the microbial community is greatly depleted which, in concert with resource use efficiency, increases the probability of spoilage dominated by a single best competitor (Setala and McLean, 2004). It has subsequently been recognized that only a few microorganisms are capable of sufficient proliferative efficiency to become the predominant spoilage organism in a given system (Mossel and Ingram, 1955; Thompson, 2009). The association of specific spoilage fungi with particular food products and processes is essential for the advancement of quality control strategies (Pitt and Hocking, 2009). Identification of specific spoilage fungi has improved greatly since the 1990's due to the advent of molecular methods and international taxonomic consensus. The identification of a limited subset of fungi chiefly responsible for food spoilage in a specific product enables the

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development of targeted prevention and intervention strategies which reduce food waste and protect quality (Filtenborg et al., 1996).

1.2. Processed foods as an ecological niche

Although oxygen limitation is used to control the growth of filamentous fungi, *Paecilomyces fulvus* (formerly *Byssochlamys fulva*) and *P. niveus* (formerly *Byssochlamys nivea*), *Penicillium expansum* and *P. roqueforti, Galactomyces* spp., and *Xeromyces bisporus* are capable of growth with less than 3% oxygen tension (Pitt and Hocking, 2009; Scholte et al., 2004). In hot-filled, shelf-stable products, vacuum is employed as a mold inhibitor, but some spoilage fungi have overcome this challenge due to the availability of residual oxygen. This risk is elevated in products with weaker vacuums and slightly higher total package oxygen due to lower fill temperatures, viscous matrixes, and packages which lack hermetic seals. Acid and water activity (A_w) are conventional formulation controls which generally inhibit bacterial growth, but fungal growth occurs over a wider range of physiochemical conditions than does growth of most spoilage-relevant bacteria. Fungi grow in food products from pH < 2 (organic acids) to > 9 (mineral waters) and water activities from 0.61 to 0.99 (Snyder et al., 2018a). In fact, several fungi can utilize organic acids as carbon sources, subsequently increasing the pH of the product, promoting bacterial growth. Weak acid preservatives, benzoate and sorbate, can also be broken down by fungi and their use selects for intrinsically resistant species.

Osmotolerant/-phyllic and xerotolerant/-phyllic yeast and filamentous fungi abound and delimit the biological extremes for proliferation in A_w-controlled environments. The identity of the solute impacts the ability of a given species to replicate. Yeast are better able to spoil high sugar solutions, compared to high salt or desiccated products of the same A_w. *Zygosaccharomyces bailii*, a common spoilage yeast, is somewhat nutritionally fastidious and can only utilize certain pentose and hexoses and requires a supplemental nitrogen source decoupled from its carbon source. Filamentous fungi have been recovered from sea salt and *Xeromyces* can replicate in media with a A_w as low as 0.61 (Grant, 2004). Filamentous fungi are also known for their ability to withstand temperature extremes. The sexual spores of some ascomycetes tolerate extreme thermal processing, surviving even the conditions used in generating high acid, shelf-stable foods that are "commercially sterile," or so called. Additionally, the refrigeration temperatures used to limit the growth of most microorganisms select for psychrotolerant bacteria and fungi. The restrictive growth conditions employed in food production impede the native microbiota, if they survive processing. The near-barren nature of these products allows those microbial contaminants which can replicate, to replicate without facing a high level of competition (Snyder et al., 2018a).

As a consequence of these highly selective conditions, often only one or a few microbes are able to contaminate and replicate to cause spoilage in a given product. These organisms have variably been referred to as the "critical fungi" (Filtenborg et al., 1996), the "associated biota", "spoilage organisms of concern," or the "specific spoilage organism" (Manios et al., 2014). These determinations have become increasingly apparent as a limited spectrum of product-specific spoilage microbes was revealed as classical morphological identification was supplanted by molecular typing (Frisvad and Filtenborg, 1993). Even in less restrictive food systems (high pH and A_w, minimal thermal processing) like fluid milk and raw meat, spoilage is often associated with the accession of a single group of best competitors and organoleptic spoilage is accompanied by decreased species richness and evenness (Doll et al., 2017). Crucially, the ability to recover a microbe from a food product alone does not represent the ability of the microbe to spoil the product (Snyder and Worobo, 2018b).

In this study, spoilage isolates were collected from commercially processed food products, along with data on the intrinsic and extrinsic formulation and process strategies, in order to determine product and process specific information predictive of spoilage fungal genera.

2. Materials and methods

2.1. Microbially spoiled commercial food products

In the present study, 127 microbial spoilage isolates were obtained from as many spoiled products submitted for evaluation through process authority and food microbiology extension programs between August 2015 and May 2017. All products were commercially manufactured by > 50 different companies and submitted for evaluation by the processor when spoilage was identified. Raw ingredients or minimally processed (e.g. fluid milk) agriculture products were not considered. "Spoilage" was determined by the manufacturer as a quality deviation in the product specifications that rendered the food unacceptable for consumption. The spoilage characteristics in products received from manufacturers included visible mycelial development, turbidity in liquid products, gas production, and organoleptic fault. Once received, products were stored under refrigeration at 4 °C for up to 48 h until evaluated as described below.

Products were grouped into nine product categories: juice/acidic beverage (n = 51), fermented (n = 12), baked good (n = 6), fruit preserve (n = 9), dried cereal and nuts (n = 8), confection (n = 13), refrigerated ready-to-eat (RTE) (n = 18), butter and oils (n = 6), and tomato-based sauce (n = 4), as similarly described by Filtenborg et al. (1996).

Thermally processed fruit and vegetable products are over-represented in this collection based on the specialization of the food microbiology extension programs which received samples. Subsequently, 40% of the products belonged to the "juices/acidic beverage" category and 36% of the collection was manufactured using hot-fill. The juice/acidic beverage category contained 100% juices, juice blends, and other acidic beverages which were either subjected to a kill step (thermal pasteurization, ultraviolet light, and high pressure processing) and refrigerated or hot-filled/bottle pasteurized and shelf-stable.

The baked good, dried cereal and nuts, confection, and butter and oils categories were all thermally processed and shelf-stable due to sufficiently low A_w. The fruit preserve and tomato-based sauces (i.e. marinara, salsa, taco sauce) categories were thermally processed and hot-filled rendering them shelf-stable. The refrigerated RTE category contained a variety of products and ingredients, but all were perishable. While the ingredients used in formulation of the refrigerated RTE foods were either thermally processed or microbiologically inert, through combination and handling during production, the final product was not stable outside of temperature control.

2.2. Identification of intrinsic and extrinsic microbial controls

For each of the spoiled products, data was collected on the physiochemical properties and associated processing conditions from the manufacturer. Critical factors used in processing were recorded as a single temperature value assigned to represent the maximum temperature of the thermal treatment for each product in its most stable condition. For products which were cold filled, the temperature was recorded as 25 °C, while the fill temperature was used for hot-filled products. The baking temperature was used only if the A_w of the product was reduced below 0.61 during baking, otherwise it was treated as a "cold filled" product due to the potential for contamination and growth of spoilage fungi following the thermal process. Deviations to the scheduled process, if known, were reflected in the assignment of thermal treatment values.

Intrinsic microbial controls associated with product formulation were also determined. The final pH of each product was determined using a pH meter (Accumet Basic AB15, Fischer Scientific, Pittsburgh, PA) and the A_w was determined using a water activity meter (AquaLab 4 TE, Decagon Devices Inc., Pullman, WA). The oxygen availability in the container was assessed by sorting each product into one of two

Table 1
Identification of process failures and associated spoilage incidents.

Problem	Product and Organism	Commercial Spoilage Example	Analysis
Problematic Raw Ingredients	Apple juice concentrate, *Alicyclobacillus* and *Paecilomyces* (*Byssochlamys*)	Contaminated fruit used to produce concentrate. Spoilage resulted when diluted back to single strength juice.	High volume detection methods; verification of the COA
	Cheese, *Mucor*	Contaminated fungal-synthesized enzyme used in production of the fresh cheese lead to hyphal growth.	Isolation of *Mucor* from enzyme stock and also spoiled product
	Cherry juice, *Fusarium*	Repeated detection of the same isolate from product made using the same fruit supplier.	Tracking trends in microbiological testing results
Post-Processing Contamination	Tomato sauce, *Penicillium*	Mycelial development environmental contamination during the fill step.	Cursory check of heat tolerance from isolated fungus
	Yogurt, *Mucor*	Product contamination due to poor sanitary design followed by temperature abuse.	Challenge studies under various production scenarios
	Yogurt, *Candida*	Product contamination due to ingress of yeast from the environment into the fermentation vessel.	Identification of GMPs violations and sanitary design short -comings
Under Processing	Shelf stable fruit juice, *Penicillium*	Pellicle development in the headspace occurred because the minimum fill temperature specified in the scheduled process was not achieved.	Review of production records from toll processor and lack of appropriate corrective action
	Pumpkin butter, *Aspergillus* (*Eurotium*)	Maximum pH specified in the scheduled process was exceeded, resulting in a decreased cumulative lethality.	Review of production records and lack of appropriate corrective actions
	Granola, *Penicillium*	Water activity of the final product was too high to maintain shelf-stability.	Comparison of a_w to reported growth requirements
Packaging Failures	Raspberry jam, *Paecilomyces* (*Byssochlamys*)	A sufficient vacuum was not achieved during production which elevated total package oxygen.	Vacuum strength assessment using canner's vacuum gauge
	Vegetable oil, *Aspergillus*	Condensation due to temperature fluctuations created a local environment with elevated $A_{w.}$	Destructive product evaluations and review of production conditions
	Caramel sauce, *Cladosporium*	Product spill around package seal created conduit for environmental contaminants.	Evaluation of container integrity

categories: high oxygen tension or reduced oxygen tension based on the packaging conditions. Products with reduced oxygen tension included vacuum sealed, hot filled, high acid foods; cold filled, hermetically sealed, fermented foods; and vacuum packed products. When an unopened container of product was provided by the manufacturer, vacuum strength (inches Hg) was measured using a Canners Vacuum Gauge with a rubber collar (10816-00 vacuum gauge, Wilkens-Anderson, Chicago, IL).

2.3. Isolation of fungi from spoiled product

Isolation of fungi from solid foods was performed by removing contaminated portions of spoiled product and placing mycelia sections near the center of a Petri dish containing non-acidified Potato Dextrose Agar (Becton, Dickinson and Co., Franklin Lakes, NJ). Although a range of growth media for culturing fungi from various environments exist, PDA was selected as an all-purpose growth substrate (Sperber, 2009). Additionally, the collection is overrepresented with spoiled juice/acidic beverages for which PDA is well suited for culturing the causative fungus. For products with low A_w, which may have selected for spoilage organisms less well suited for growth on PDA, confirmation of recovery was supported by morphological examination as mycelial development on spoiled products could be visually identified. For liquid products and those without visible mycelial development (e.g. yeast spoilage), a product sample (10 g) was serially diluted in 0.1% peptone water (Becton, Dickinson and Co., Franklin Lakes, NJ) and plated on PDA. Depending on the product, low levels of various background biota appeared on the lower dilutions. However, the predominant spoilage microorganism was identified at the 10^{-3} dilution or greater. Plates were incubated at 25 °C for seven days, and isolates were then subcultured and re-incubated. For filamentous isolates, pure cultures were incubated for up to 30 days to facilitate spore development. Freezer stock was prepared for long-term storage (−80 °C) in 20% glycerol. Rarely (6%), bacteria were identified as the causative spoilage agent. In the case of *Alicyclobacillus* spp. (n = 1 in the collection), colonies appeared on PDA and the identity was confirmed through sequencing of the 16S region. In all other cases (n = 7), when fungi could not be isolated from the product, additional testing on Trypticase Soy Agar (Becton, Dickinson and Co., Franklin Lakes, NJ) lead to the identification of the bacterial spoilage organism. By virtue of the processing and storage conditions, pH, and A_w of the products involved in this study, the vast majority of the spoilage agents were yeast and filamentous fungi.

2.4. Identification of fungal isolates

Genus-level identification was made by sequencing the ITS barcode region. Isolates were grown on PDA and fungal DNA was extracted from scraped colonies using the PowerSoil DNA isolation kit and following the manufacturer's instructions (Qiagen, Hilden, Germany). For filamentous fungi, the internal transcribed spacer region at ITS 1 and 2 flanks the 5.8S gene was amplified using the Primers ITS4 (5′TCCTCC GCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACA AGG-3′) as described by White (1990). For yeast, the D1/D2 domain of the 26S rDNA gene was amplified using the Primers NL1 (5′-GCATAT CAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GCTCCGTGTTTCAAGA CGG-3′) as described by Herzberg et al. (2002). PCR products were confirmed by gel electrophoresis, purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced by the Cornell University Life Science Core Laboratories Center (Ithaca, NY) using an ABI PRISM 3730 DBA sequencer (Applied Biosystems, Foster City, CA). The resulting sequence was compared to the NCBI BLAST nucleotide database, excluding uncultured microbes. The sequences have been submitted in Genbank under the submission number SUB4201409. Any bacterial spoilage was only identified as “Bacteria” within the data set.

Isolates with food industry-relevant teleomorphic names were so noted as they are functionally distinct from other propagules based on their capacity to survive heat processing, as in the case of *Neosartorya* (*Aspergillus*) and *Byssochlamys* (*Paecilomyces*), and *Eurotium* (*Aspergillus*). The teleomorphic names are still recognized and used by the food industry, therefore they were retained for clarification in this study. Assignments were based on (Houbraken and Samson, 2017). In each of these cases, the name of the food-spoilage relevant form, commonly used within the industry, was applied in addition to the holomorphic genus name. For the statistical analysis, all mucoralean isolates (*Mucor, Rhizopus,* and *Syncephalastrum*) were grouped together.

2.5. Root cause analysis of select quality failures

Several of the spoiled products (n = 11) were part of short and long-term studies (e.g. Snyder et al., 2016; Biango-Daniels et al., 2018; Snyder and Worobo, 2018b) to determine the environmental source, process failure, or etiological agents associated with notable quality deviations. The full descriptions of spoilage issues for which a root cause was determined are included in Table 1. For root cause analyses that required the differentiation between post-process contaminants and heat resistant filamentous fungi, cursory thermal destruction tests were implemented to assess heat resistance as adapted from (Samson, 2010). Briefly, 30-day old plates were scraped with phosphate buffered saline (pH 7.2) and transferred to a thin-walled plastic bag (Whirl-Pak, Uline, Pleasant Prairie, WI) and heat sealed. Bags were submerged in a hot water bath at 70 °C for up to 5 min, and a single bag was pulled every minute. Heat-treated samples were immediately submerged in an ice bath, serially diluted, and spread plated on PDA for enumeration of survivors. Although this method does not definitively prove heat resistance, it does allow for rapid identification of post-processing contamination for isolates which could not possibly survive product-relevant thermal processing conditions. For confirmation that a specific isolate was the cause of a given spoilage defect, Koch's postulates were applied (Sperber, 2009). The putative spoilage agent must have been cultured from the spoiled product and grown in pure culture for identification as described above. The putative spoilage isolates were then reintroduced into fresh product and spoilage characteristics were observed before re-isolation of the spoilage agent. Introduction of the putative spoilage agent was made under conditions specific to the relevant point of production.

2.6. Data analysis

The continuous predictor variables of pH and A_w were transformed into categorical variables for the data visualizations in Fig. 1. Low pH and high pH categories were delimited based on the regulatory cutoff for inhibition of *Clostridium botulinum* germination at pH 4.6. Water activity levels of low (< 0.6–0.85), intermediate (0.85–0.95), and high (> 0.95) based on cutoffs specified in Sperber (2009) which are closely related to those used in food safety regulations, $A_w < 0.93$ inhibits *C. botulinum* germination, and $a_w < 0.85$ inhibits *Staphylococcus aureus* toxin production, the lowest A_w that supports the growth of foodborne bacterial pathogens. Although these categorizations are framed largely around the growth potential of bacterial pathogens, these cutoffs are, for this reason, often targeted as critical limits by food processors. Therefore, grouping spoilage according to the convention utilized by manufacturers is most likely to result in useable information.

Statistical analysis was performed using R statistical software (version 3.0.1, R-project, Vienna, Austria). The bipartite network analysis was developed using the R package ggbipart (Fig. 2) and a heatmap (Fig. 3) was built using the heatmap function in ggplot. A classification tree (Fig. 4) was constructed using the rpart package. Partitions were made based on the predictor variables of product category, process regime, pH, and A_w for genera isolated with at least four unique observations. The yeast genera remaining were collectively assigned to a single categorical outcome “yeast” which reduced the overall number genera in the model to ten in a data set containing 117 observations. In construction of the classification tree, a split rule requiring at least 20 observations to create a subsequent branch was included to minimize over fitting. At terminal nodes, outcomes were listed for all genera with a probability of 0.15 or greater.

3. Results and discussion

3.1. Diversity of fungal spoilage isolates

A single causative spoilage isolate was identified from each product and the total background biota was not assessed as in Garnier et al., 2017; Al-Bulushi et al. (2017), and De Clercq et al. (2015). Spoilage organisms (n = 127) were isolated from commercially manufactured products, of which 9 were determined to be bacteria, 26 were yeast, and 92 were filamentous fungi. All of the isolated fungi belonged to the phylum Ascomycota, except for seven *Rhodotorula* isolates (Basidiomycota) and four mucoralian isolates from the genera *Mucor, Rhizopus,* and *Syncephalastrum* which were the most distantly related isolates from the majority of fungi in this study (Snyder and Worobo, 2018b). Eight unique yeast genera were identified and included *Rhodotorula, Wickerhamomyces* (*Pichia), Candida, Torulaspora, Saccharomycopsis, Aureobasidium, Saccharomyces,* and *Exophiala*. The vast majority of these isolates belonged to the family Saccharomycetaceae, in addition to one isolate from each of the families Aureobasidiaceae and Herpotrichiellaceae. Of the ascomycete filamentous fungi identified the 14 genera belonged to two major groups, Eurotiomycetes and Dothideomycetes. Of those 92 filamentous isolates, 42% were from the Trichocomaceae family. Some genera were only isolated once and these included *Monascus*, *Epicoccum*, *Talaromyces,* and *Sarocladium*. Other genera were isolated repeatedly. The occurrence of filamentous fungi which appeared multiple times ranged from 2% (*Phoma*) to 18% (*Penicillium*). Around half (57%) of isolates came from low pH (< 4.6), and high A_w (65%) products (Fig. 1), common physiochemical conditions in foods. Moreover, the majority of isolates were obtained from high A_w (> 0.95) products due to the abundance of juices within the data set. The products evaluated were manufactured from fruits and vegetables (49%) and included, juices, preserves, sauce/relish, and fermented hot sauces; dairy (5%) and included yogurt, cheesecake, and cheese; and nuts and grains (16%) and included granola, cookies, cake, nuts, seeds, sunflower oil, and tempeh.

The most frequently occurring genus was *Penicillium* (n = 23) which was isolated from all processing conditions and all product categories except fruit preserves - although this may be due to the low number of observations for this category since Penicillia have been reported to cause spoilage of these products (Thompson, 2009). *Penicillium* is one of the most prevalent spoilage molds (Sharpe and Pettipher, 1983). Of the 23 *Penicillium* isolates identified, 10 were post-processing contaminants in hot-filled products while the remaining 13 isolates were from pasteurized or non-thermally processed products, which indicates the diversity of conditions under which Penicillia were capable of causing spoilage.

Yeast were isolated from all product types except fruit preserves, tomato-based sauces, and dried cereals and nuts, and from all process conditions except hot-fill. This is aligned with reports of yeast heat sensitivity and association with high A_w environments. Although yeast can spoil concentrated sugar solutions (confection products), they are less common in dry or high salt solutions (Wang et al., 2015). Yeast are responsible for 75% of acidified food spoilage (Sharpe and Pettipher, 1983) but are heat sensitive. Subsequently they were readily isolated from pasteurized juice (11 of the 26 yeast isolates were from spoiled pasteurized juices), but not from hot-filled, shelf stable juices. *Candida* was the most frequently isolated yeast genus followed by *Rhodotorula*, *Wickerhamomyces* (*Pichia),* and *Torulaspora*. Rarely, yeast were isolated from baked goods and oils (n = 3). Because of the reduced A_w, baked goods were more frequently spoiled by filamentous fungi (71%) but some cases of spoilage by yeast and bacteria have been reported (Sperber, 2009). *Saccharomycopsis* has been isolated from oils used as processing aids in bread production accounting for over half of the spoilage isolates present in blade oil (Legan and Voysey, 1990) and in this study, may have resulted from condensation in container while product was warm.

3.2. Association of fungal genera with process regimes and product category

Fungal spoilage genera are associated with process regime (Fig. 2) and product category (Fig. 3). Although these predictors are sometimes

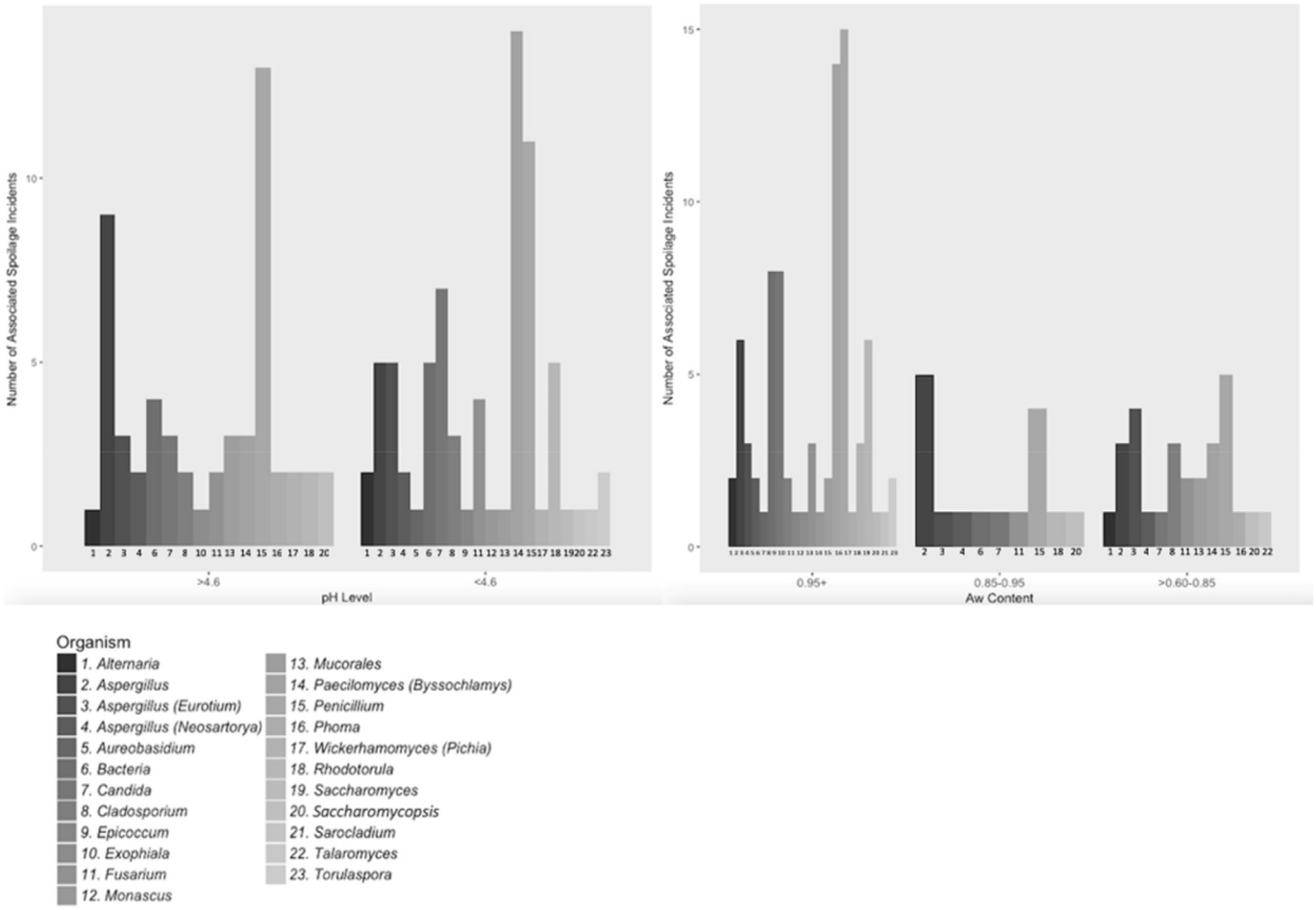

Fig. 1. Distribution of spoilage fungi by genus in products of high and low pH (A) and high, intermediate, and low water activity (B). Numbers (1–23) represent fungal genus.

related (e.g. fruit preserves were always hot filled), there were many cases when they diverged (e.g. juice/acidic beverages were either hot-filled or pasteurized), which impacted the identity of associated spoilage fungi. The network analysis shown in Fig. 2 illustrates the number of observations for each identified fungal genera under given processing conditions. Pasteurization was least selective and 15 different genera were capable of spoiling products manufactured under this processing regime. This process category was almost entirely composed of refrigerated juices/acidic beverages.

Hot-filled products (juices, fruit preserves, tomato-based sauces, confections) were spoiled by Heat Resistant Mold (HRM) [*Paecilomyces* (*Byssochlamys*), *Aspergillus* (*Eurotium*), *Talaromyces,* and *Aspergillus* (*Neosartorya*)] in addition to other filamentous fungi (*Penicillium, Monascus, Fusarium*) which indicated that these products were susceptible to both contamination of ingredients with HRM as well as spoilage from post-processing contamination or under-processing. In fact, only about half of the isolates obtained from hot-filled products were HRM, which is likely an over estimation of the isolation frequency for these organisms generally in commercially spoiled products. This suggests that environmental contamination is likely to be a cause of spoilage despite manufacturers' frequent concerns to the contrary (Table 1). The most frequently isolated HRM was *Paecilomyces* (*Byssochlamys*) with 16 observations. Ingredients are the most common source of *Paecilomyces* (*Byssochlamys*) and post-processing contamination of HRM is less likely to result in spoilage since the thermal process is often essential in initiating germination of the ascospores (Rico-Munoz, 2016). Yeast were associated with pasteurized, fermented, and non-thermally processed products but not hot-fill. No spoilage yeast relevant to the food industry is known to produce highly heat resistant ascospores (Snyder et al., 2018a). Baked products were infrequently spoiled by yeast since the minimum A_w required to support yeast growth is often exceeds those typical of baked products. HRM were almost exclusively isolated from hot-filled products, with the exception of a single *Paecilomyces* (*Byssochlamys*) isolated from spoiled cheesecake. Houbraken et al. (2008) reported the detection of a single *Paecilomyces variotii* (*Byssochlamys spectabilis*) isolate from spoiled rye bread in a collection of 16 total isolates; however, that isolate lacked significant heat resistance compared to the rest of the collection and, notably, *P. variotii* produces heat resistant ascospores through heterothallic mating.

The physiochemical properties and ingredient-associated biota for product categories also select for specific spoilage genera. For example, eight *Aspergillus* (*Eurotium*) isolates were identified in this collection and were taken from products with an average A_w level of 0.82. This is in keeping with previous work by Pitt and Hocking (1977) on this xerophillic fungus. The Mucorales (*Mucor, Rhizopus,* and *Syncephalastrum*), although prolific spoilers of raw fruit and vegetables, were infrequently isolated from processed products as they are highly sensitive to heat (Snyder et al., 2016) and favor raw agriculture commodities (Pitt and Hocking, 2009). However, Mucorales were isolated four times in this study from fermented dairy products and dried nuts. Spoilage of fermented dairy products by *Mucor* and *Rhizopus* has previously been reported, as has spoilage of dried seeds by *Rhizopus* and *Syncephalastrum* (Adebajo et al., 1994; Snyder and Worobo, 2018b). Yeasts are recognized as the major cause of spoilage for yogurt because the low pH provides a selective environment and companies often employ a quality standard of < 10 CFU/g yeast and mold for finished product micro

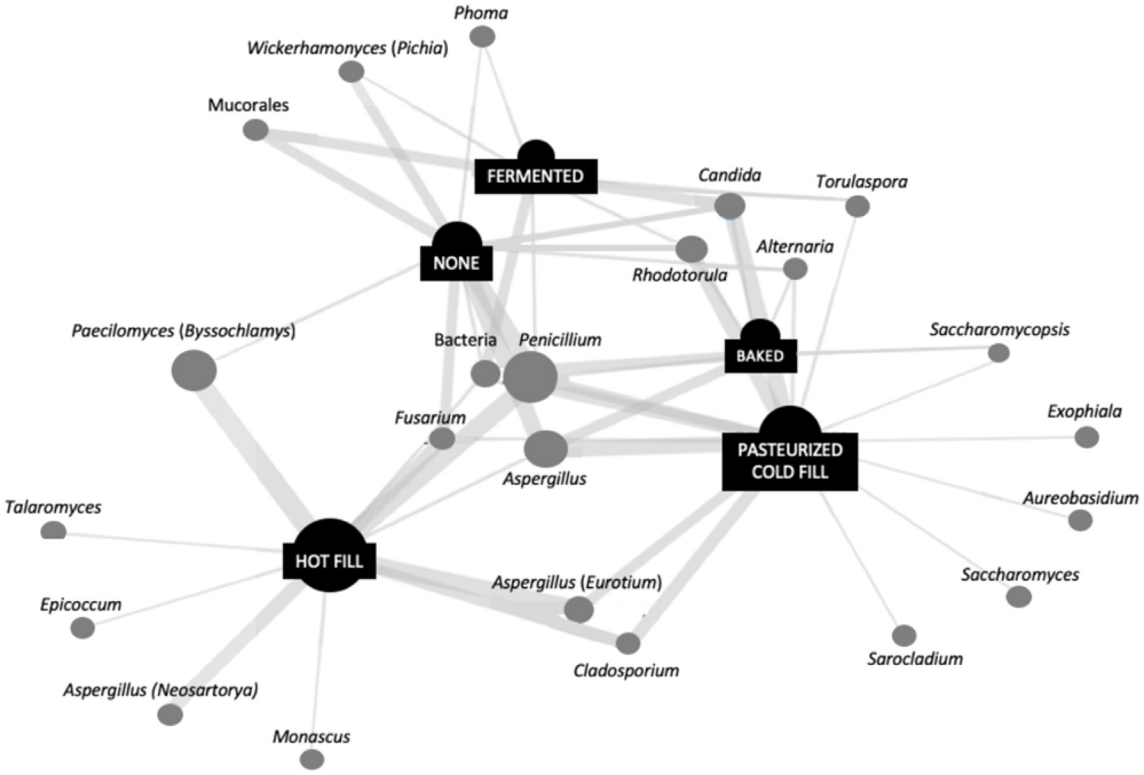

Fig. 2. Network analysis of specific spoilage organisms (gray) and processing regime (black). Radius represents number of observations of the organism or process. Line thickness represents number of spoilage incidents.

testing, while > 100 CFU/g has been associated with a decreased shelf-life (Sperber, 2009). *Candida, Torulaspora,* and *Mucor* (yeast-like phase) were isolated from yogurt within this study and were associated with sanitation issues (Table 1). More generally, Mucorales and yeast were associated with refrigerated RTE and fermented products (Fig. 3), while the Trichocomaceae were more frequently isolated from the food matrix categories of juices/acidic beverages, fruit preserves, and tomato-based sauces. However, *Penicillium* and *Aspergillus* were found across the spectrum of product categories tested. The fungal spoilage genera associated with juice, by far the largest product category in this study,

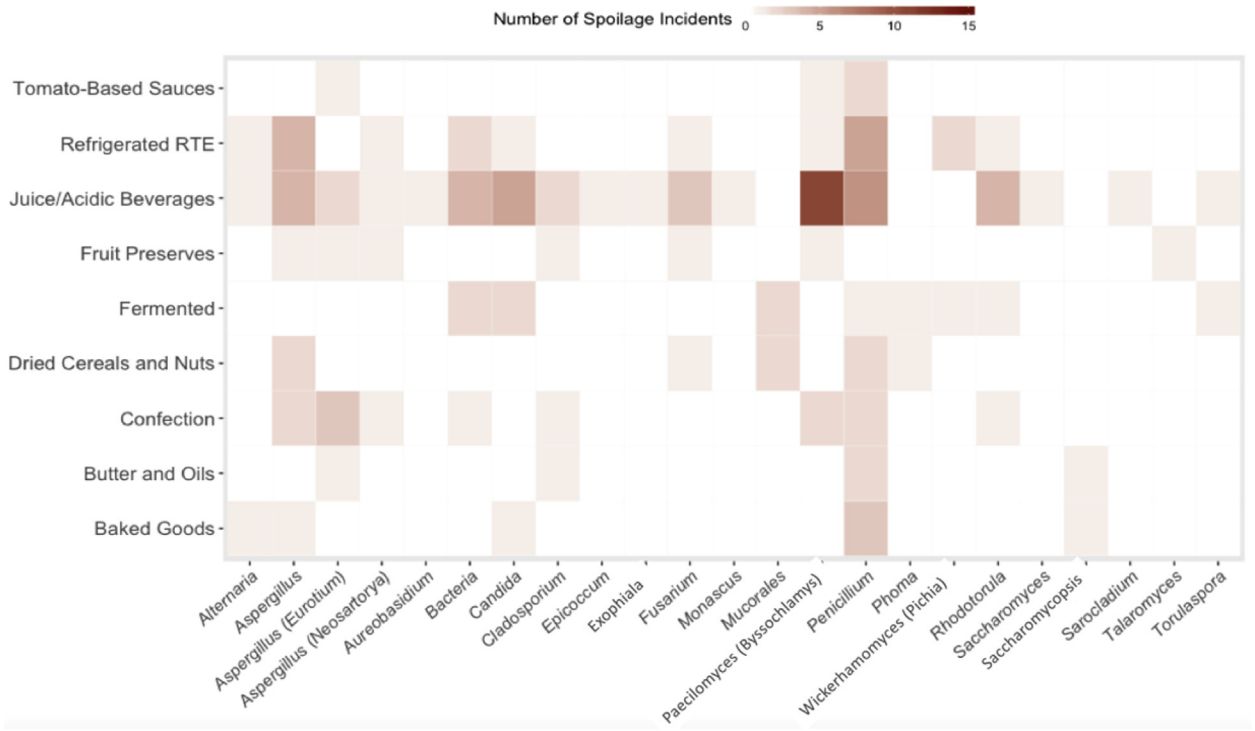

Fig. 3. Stratification of fungal genus isolated from commercially spoiled products by product category. Shaded cells represent increasing number of observations. White cells represent organism and process combinations with zero observations.

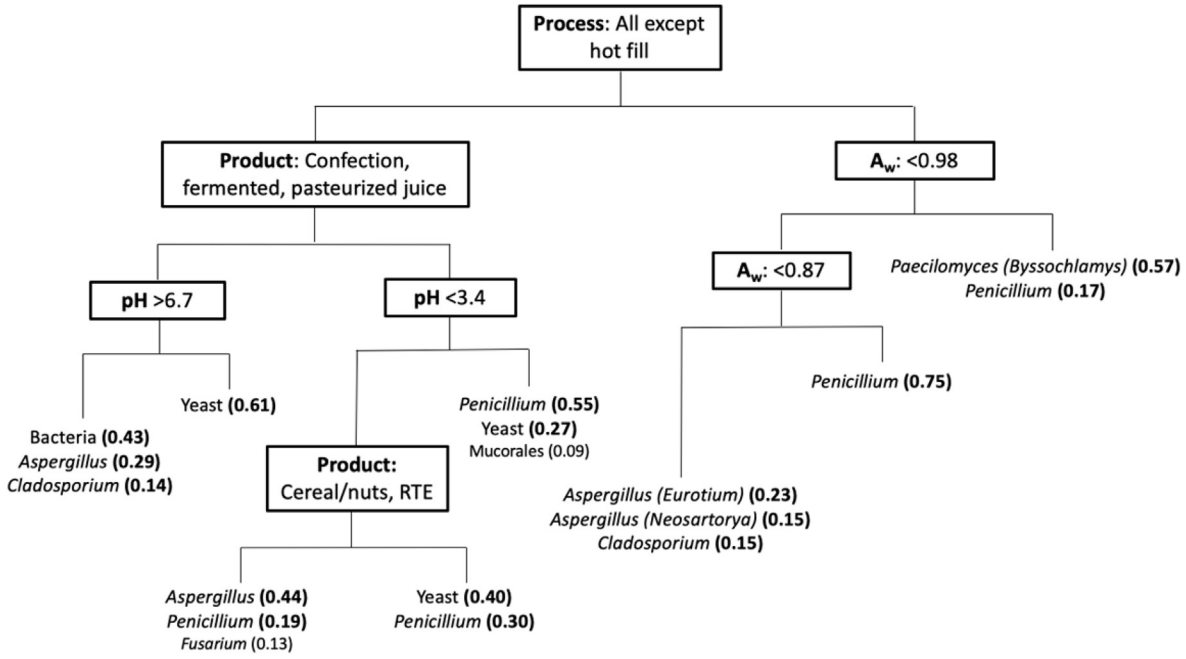

Fig. 4. Fungal spoilage genera classified based on processing conditions and physiochemical properties of the product. The criterion at each split partitions spoilage fungi into homogenous subsets, left-handed branches are enriched in observations that align with the node criterion. Probabilities for each genus at a leaf position are included parenthetically.

depended largely on the processing conditions.

3.3. Physiochemical properties predictive of dominant spoilage genera

The probability of isolating particular genera from a given product based on physiochemical attributes and processing conditions are presented in Fig. 4. These predictions were generated by recursively partitioning observations for the ten genera which had at least four observations. The first split in the classification tree is based on processing conditions, hot-fill products are directed to the right of the tree while other processing conditions are directed to the left. The hot-fill branch was enriched in HRM [*Paecilomyces* (*Byssochlamys*), *Aspergillus* (*Eurotium*), *Aspergillus* (*Neosartorya*)] while some heat sensitive fungi, like yeast, were excluded. However, other environmental contaminants, *Cladosporium* and *Penicillium,* were predicted in hot-fill products at 0.15 and 0.17, respectively. This suggests the propensity for failures in production to result in microbial spoilage of a product accounts for a sizable portion of the spoilage incidents. The probability of isolating *Paecilomyces* (*Byssochlamys*) was greatest (0.57) in high A_w, hot-filled products, likely due in part to the proportion of the juice/acidic beverages which were spoiled by this organism. Moreover, *Paecilomyces* (*Byssochlamys*) is reportedly inhibited by low A_w conditions while, in contrast, *Aspergillus* (*Eurotium*) had the highest probability of isolation from low A_w, hot-filled products (0.23) and is known for low A_w tolerance (Biango-Daniels et al., 2018).

On the left side of the classification tree, products that were pasteurized, fermented, baked, or non-thermally processed were further broken down by physicochemical condition. Confections, pasteurized juices, and fermented products were split by pH. Products with a near neutral pH (> 6.7) which included vegetable juices, cheese, and chocolate products were most likely to be spoiled by bacteria (0.43), *Aspergillus* (0.29), and *Cladosporium* (0.14). The remaining products, refrigerated RTE, cereals and nuts, baked goods, and butter and oil were spoiled by *Aspergillus, Penicillium,* yeast, and with a lower propensity, mucorales and *Fusarium* depending on pH.

3.4. Identification of production failures contributing to spoilage incidents

The product associated attributes of physiochemical properties, processing regime, and product category influence the spoilage fungal genera. However, the identity of the spoilage organism is not exclusively predictable based on these features (Fig. 4). Production failures likewise appeared to influence the spoilage outcome. As discussed above, the probability of hot-filled products spoiling due to HRM compared to post-processing contamination is an indication of the mediating influence of production failures, or inaccurate expectations that GMPs and SSOPs are either infallible or of little consequence. Root cause was identified for several of the spoilage incidents included in this study. A summary of the most common problems that lead to spoilage, and specific product and organism examples of these incidents, are presented in Table 1. In several of the cases, the product was under-processed through reduced thermal treatment or because of inadequate acidification - the pH and type of organic acid influence the accumulated lethality during thermal processing, therefore elevated pH levels increase the probability of survival for contaminating fungi (Sastry, 1986). In these cases, filamentous fungi with modestly heat resistant hyphae/asexual spores were identified as the associated spoilage organism. These Penicillia and *Monascus* isolates did not meet the conventional definition for HRM, they were inactivated by a thermal treatment of 20 min at 80 °C (Samson, 2010). However, a moderate heat tolerance contributed to the spoilage potential for these organisms, and so the industry may consider the adoption of an additional intermediate classification for spoilage fungi capable of spoiling heat-treated products when a process deviation has occurred. *Monascus* has previously been associated with moderate heat resistance (Panagou et al., 2003) as have some resistant Penicillia hyphae (Thompson, 2009).

Other elements influencing spoilage fungi that are not captured in physiochemical product properties and processing conditions included cleaning method, packaging type and method, and equipment design. Inclusion of these factors in predictions of pertinent spoilage fungi may improve quality risk assessments. In dry environments, such as those used in the production of baked goods, fats and oils, and confections,

the dry-cleaning regimen used by manufacturers may be insufficient to mitigate fungal spoilage. In instances of low A_w product spoilage, we identified low vacuum strength and the potential for elevated oxygen levels in container headspace as contributing to product spoilage, along with the potential for condensation development in A_w controlled foods (Table 1). Thermal process also intersects with total package oxygen as fill temperature directly correlates with vacuum strength as well as dissolved oxygen (Sperber, 2009). The fill step is highly susceptible to post-processing contamination. Preventing spills, condensation, and correctly forming package seams are requisites for controlling spoilage and are usually addressed through GMPs. However, the results of this study suggest that failures at this step are not infrequently the cause of spoilage incidents. Sanitation and sanitary design is another major category of production failures contributing to spoilage. Equipment design and maintenance as well as environmental sanitation influences the rate of post-processing contamination. Effective preventive maintenance programs and verification of these sanitation systems may be useful in production environments which rely on these programs for quality control.

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Appendix A. Supplementary data

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