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Population Changes of Indigenous Murine *Candida pintolopesii* under Various Experimental Conditions and Routes of Inoculation

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Fecal *Candida pintolopesii* population levels were found to be significantly affected by laparotomy-inoculation-Bollman apparatus restraint, laparotomy-inoculation, and a milk diet. Gastrectomized rats could not support yeast populations; in intact animals, yeast cells failed to colonize the gastrointestinal tract distal to the stomach. Copraphagia contributed little to stomach yeast populations, supporting the notion that stomach yeast growth occurs at fairly rapid rates.

Microorganisms are found in the gastrointestinal (GI) tracts of virtually all conventional animals in concentrations ranging from 10^9 in the upper intestine to 10^{13} in the lower intestine and colon (9). Commonly found in the tracts of rats and mice are indigenous yeasts capable of populating certain sections of bowel. In particular, *Candida pintolopesii* (formerly *Torulopsis pintolopesii*) forms layers on the secreting epithelium of the stomach (10). It is a major microorganism that can be isolated from all sections of the GI tract (4, 8).

In vitro studies have been done on this yeast (1, 3), yet there is a scarcity of information of in vivo yeast population changes. Previous studies have provided data about microbial populations at given times; however, the effects of changes that occur from circadian rhythms, dietary factors, or environmental factors are not known. The question has been raised regarding the importance of attachment phenomena in maintaining yeast microbial populations. These studies attempt to define colonization and/or population changes of *Candida pintolopesii* in microenvironments extraneous to the stomach for the purpose of determining yeast population growth within the stomach. They also show the influence of environment and diet on yeast populations.

MATERIALS AND METHODS

Total and partial gastrectomy and yeast recovery from various levels of the digestive tract. Fifteen yeast-colonized male Sprague-Dawley (180 to 400 g) rats (Harlan Sprague Dawley, Madison, Wis.) were housed on pine shavings in shoebox cages in a conventional room. The room temperature was kept from 68 to 78°F and had a 12-h light–12-h dark cycle. Five rats were totally gastrectomized (6), five rats had forestomach gastrectomies (6), and five served as controls by having nothing done to them. All animals were weighed both at the beginning and end of the study. Originally isolated from these animals, *Candida pintolopesii* was orally inoculated into them 5 days postoperatively by a 24-h yeast-peptone-glucose broth (Difco) culture (10^9 yeast CFU) poured into the drinking water. Yeast cells remained in the water for 24 h to preclude any effect the surgery may have had on yeast levels. Fourteen days postinoculation (p.i.), the animals were sacrificed and portions of the GI tract and its contents were collected and weighed. The glandular stomach and nonglandular stomach were removed, gently cleared of their contents, and weighed; then 2 cm of jejunum was collected and weighed. All of these tissues were dropped into individual grinding tubes containing a sufficient amount of sterile water to make a 1:10 dilution (weight/volume) and ground with a tissue grinder (American Scientific Products) to form a homogeneous suspension. Cecal contents (0.3 ml) and a weighed fecal pellet from the terminal colon were also diluted 1:10 (volume/volume and weight/volume, respectively) with sterile water. Estimates of the yeast population levels in the suspensions were made by serial dilution and spread plate techniques on yeast-peptone-glucose agar (YPG agar; Difco) supplemented with 40,000 U of penicillin and 0.04 g of streptomycin per liter at 37°C.

Yeast colonization following different routes of inoculation. Twenty-eight yeast-free male and female Sprague-Dawley rats (180 to 400 g) (Sasco, Omaha, Neb.) were housed singly in wire-bottomed cages in a conventional room. A chronic indwelling catheter was made from silastic tubing connected to an injection port (Abbott) with cyanoacrylate (Loctite Corp.). This was disinfected in 70% ethanol for 48 h and surgically implanted into the duodenum of 20 animals and the jejunum of the remaining 8 animals. Total volume in the catheter was 0.1 ml. The injection port was placed in the subcutaneous tissue of the flank with the tubing entering the abdomen and subsequently the intestinal lumen. The intestinal entry point was sutured to the abdominal wall. Animals were allowed to stabilize postoperatively for a week, and twice a day handling was done at 8-h intervals. Total fecal output was collected and weighed daily. Forty-eight hours prior to the start of the experiment, feces were checked for yeast cells. During this time, animal weights were recorded every other day and then taken every day at the beginning of the experiment. The experiment began between 2 and 4 p.m. on Thursday. This time was chosen because there was minimal activity in the room on Friday and the weekend.

Group A consisted of six control animals with intraduodenal catheters. These were inoculated with *Candida pintolopesii* 3 days postoperatively by a 24-h yeast-peptone-glucose broth culture (10^9 yeast CFU) poured into the drinking water. Yeast cells remained in the water for 24 h, after which time the water was changed. The culture was isolated and characterized from Sprague-Dawley rats used in study 1. After a 7-day postoperative equilibration period, three rats received 0.1 ml of saline by gastric gavage, and three rats received 0.1 ml of saline into the implanted catheter with 0.12 ml of air infused into the catheter to evacuate its contents into the duodenum. The total fecal output was collected every 8 h, weighed, and diluted 1:5 (weight/volume) in sterile water. Estimates of yeast popula-

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TABLE 1. Estimated yeast populations in gastrectomized and forestomach-gastrectomized rats

Rat group	Mean CFU/g or ml ± SD (n = 6)				
	Glandular stomach	Forestomach	Jejunum	Cecum	Colon (pellet)
Control	$4.88 (\pm 1.36) \times 10^6$	$1.22 (\pm 2.04) \times 10^6$	$4.33 (\pm 3.70) \times 10^6$	$2.00 (\pm 1.61) \times 10^7$	$2.28 (\pm 2.29) \times 10^7$
Forestomach gastrectomy	$2.54 (\pm 0.72) \times 10^6$	—[a]	$4.12 (\pm 2.47) \times 10^5$	$8.45 (\pm 5.59) \times 10^6$	$1.90 (\pm 1.90) \times 10^7$
Gastrectomy	—	—	0	0	0

[a] Organ was surgically removed.

tion were made by serial dilution and plate technique. Cultures were done on YPG agar with 0.06 g of gentamicin per liter of medium at 37°C to determine total fecal yeast CFU per 8-h period. Total yeast CFU per gram of feces were also calculated at this time. After 48 h, the animals were sacrificed. The entire stomach contents, first half of the small intestinal contents (SI-1), entire second half of the small intestinal contents (SI-2), entire cecal contents, and entire colonic contents were weighed and diluted 1:5 in sterile water, with the division between SI-1 and SI-2 calculated to be the midpoint between the pylorus and the cecum. To acquire this sample, the intestinal contents were extruded and assayed. The emptied tissue was weighed and made into 1:10 (weight/volume) solutions with sterile water. Estimates of the yeast population levels were performed in the same manner as for total fecal output. Tissues were ground to make a homogeneous suspension with a tissue grinder. It was determined that from 95 to 99% of the yeast counts were present in the SI-1, SI-2, cecal, and colonic extruded gut contents but not in the tissue when ground and cultured. Group A rats were sampled at 48 h p.i. to determine whether changes in fecal yeast levels would occur from repeated handling.

Crude stomach fraction derived from Group A and postexperiment yeast-inoculated groups C2 and D was used as the inoculum for groups B1, B2, C1, C2, and D as described below. Stomach tissue homogenate was filtered through gauze and centrifuged at 1,350 × g for 8 min at 37°C with a portion of the pellet used as the inoculum. The time from the sacrifice of the rats used to prepare the stomach fraction to the inoculation of groups B1, B2, C1, C2, and D was approximately 35 to 45 min.

Due to the low yeast counts in the tissues of the rats in group A, only the gut contents were sampled for yeast levels in SI-1, SI-2, cecum, and colon in groups B1, B2, C1, C2, and D. Unlike the samples from group A, the stomach mucosa was scraped with a dull scalpel blade and pooled with the stomach contents in the other groups.

Group B consisted of six rats with intraduodenal catheters. After a 7-day postoperative equilibration period, this group received 0.1 ml of a crude stomach fraction (10^3 to 10^6 yeast CFU) by gastric gavage. Total fecal output was collected every 8 h and cultured as described for group A. Four rats were sacrificed at 16 h p.i. (group B1), and the remaining two were sacrificed at 24 h p.i. at (group B2) in order to measure the increase of yeast population levels in the stomach.

Group C consisted of eight rats with intraduodenal catheters. This group received 0.1 ml of the crude stomach fraction (10^3 to 10^6 yeast CFU) through the implanted intraduodenal catheter with 0.12 ml of air infused to flush the catheter contents into the duodenum. Total fecal output was collected every 8 h and handled as described for group A. Five rats (group C1) were sacrificed at 24 h p.i. and gut contents were sampled as described for group B. The remaining three rats (group C2) had total fecal output sampled at 8 and 16 h p.i. and a single fecal pellet was sampled at 72 h p.i.

Group D consisted of eight rats with intrajejunally implanted catheters. These rats were inoculated with 0.1 ml of the crude stomach fraction (10^3 to 10^6 yeast CFU) through the implanted catheter with 0.12 ml of air infused to flush the catheter contents into the jejunum. Total fecal output was collected, weighed, and cultured every 8 h for 24 h in the same manner as for group A animals. A single fecal pellet was then sampled at 72 h p.i.

Effects of restraint surgery, and nutrition on yeast population levels. Nine male Lewis rats (200 to 400 g; Charles River) were placed in a Bollman apparatus (a restraint device that allows animals to move but not to turn around) for an 18-day period, gradually increasing their restraint time to a maximum of 48 h. Food and water were offered ad libitum, and animals were weighed daily. Two days after the 18-day adjustment period, rats were given laparotomies, gastrically inoculated with 0.1 ml of saline, and placed in the apparatus for 48 h. Animals were exercised and total fecal output was collected every 8 h. Pretest and postadaptation fecal yeast levels were measured, and total yeast output was assayed as in group A of the colonization experiment. Laparotomy was performed with halothane anesthesia and took about 20 min. Five animals were sacrificed and sampled as described for group B1. The remaining four animals' fecal yeast levels were monitored periodically for a 2-month period, at which time these four rats and four male Sprague-Dawley rats were placed on a milk diet for 72 h. Quantitative fecal yeast CFU were measured, and all rats were weighed at 1, 2, 3, 5, 7, 14, 21, and 28 days. At 28 days, those animals which lost yeast cells in the stool were inoculated with *Candida pintolopesii* by a 24-h yeast-peptone-glucose broth culture placed in the drinking water. Feces were checked 1 week later for colonization by inoculating a fecal suspension onto YPG agar with 0.06 g of gentamicin per liter at 37°C.

Four yeast-colonized male Lewis rats were given laparotomies and gastrically inoculated with 0.1 ml of saline while under halothane anesthesia but were not placed in the Bollman apparatus. Total fecal output was collected every 8 h for 48 h, and the intestinal contents were collected and assayed as for animals in group B of the colonization experiment.

Analysis. All tests of significance used students ± test for unmatched pairs or the Wilcoxon signed-rank test.

RESULTS

Yeast population levels from gastrectomized, forestomach-gastrectomized, and control animals are presented in Table 1. Totally gastrectomized animals failed to harbor any yeast cells to a detection level of 5 × 10^3/g of sample. Forestomach-gastrectomized rats had fewer yeast cells in the stomach and the jejunum than normal animals. Higher yeast levels were found in the glandular stomach than in the nonglandular stomach in the control animals. Compared

with the control group during the 2-week postoperation period, forestomach-gastrectomized rats lost no weight; however, gastrectomized animals lost from 2 to 10% of body weight.

The results of yeast colonization following various routes of inoculation are presented in Table 2. The presence of the catheter, method of handling, and intraduodenal and gastric inoculation had no effect on fecal yeast population levels or on the weight of feces produced per day. Yeast population levels were comparable to those in the control group in the gastrectomy study after differences in sampling were taken into account.

Gastrically inoculated rats were readily colonized with yeast cells. Group B1 had lower levels than group B2, probably due to less time allowed p.i. before sacrifice, allowing less yeast growth.

None of the jejunally inoculated rats were readily colonized. All fecal yeast levels of group D rats fell to zero at 24 h p.i., with only two of the rats becoming colonized at 72 h p.i. Fecal yields during the first 16 h ranged from 0 to 87% of the original inoculum and averaged 26%.

All of the colonized duodenally inoculated animals (group C) had stomach yeast levels at 16 h p.i. that were comparable to those of group B. Half of the rats failed to become colonized.

None of the rats lost weight during the colonization study.

The detection limit of YPG agar with penicillin and streptomycin was not adequate, so YPG agar with gentamicin was used. The latter medium had a detection limit of 20 per g of sample. These antibiotics did not affect yeast growth.

Fecal yeast levels were not significantly affected by the adaptation schedule, but fell from 2.4×10^7 to 1.80×10^7 yeast CFU/g of feces to 4.8×10^3 to 5.40×10^3 yeast CFU/g of feces at 40 h p.i. ($P = 0.01$, Wilcoxon test). The five animals sacrificed at 48 h p.i. did not have gastric erosions or ulcers, yet some animals lost up to 8% of body weight during the 48-h period. The four rats that were not sacrificed after the laparotomy, inoculation, and Bollman apparatus restraint had stable but lowered fecal yeast population levels (1.43×10^6 to 0.466×10^6 yeast CFU/g of feces) for the following 2 months ($P = 6.25$ Wilcoxon test). When placed on a 72-h milk diet, their fecal yeast populations were not detected for a 4-week period, at which time they were recolonized with *Candida pintolopesii*.

The four animals that were given laparotomies and inoculated had pretest levels of 2.1×10^7 to 1.6×10^7 yeast CFU/g of feces and 40-h p.i. levels of 1.53×10^6 to 3.38×10^6 yeast CFU/g of feces (not significant). They experienced a slower decrease than the group that received laparotomies, inoculation, and Bollman apparatus restraint.

Fecal yeast counts fell ($P = 6.25$, Wilcoxon test) for Sprague-Dawley rats having no previous experimental history when they were placed on a 72-h milk diet, but these levels returned to normal when the animals were switched to the rodent chow diet.

DISCUSSION

As evidenced by the fact that no yeast cells were found in totally gastrectomized animals, the existence of the stomach was essential to yeast survival. (This is supported by Savage [10].) However, the stomach may have altered the environment distal to it to allow yeast colonization and growth. The failure of yeast colonization distal to the duodenum and detection in feces of fewer yeast CFU than in the original inoculum did not support the notion that the yeast cells grew in this area.

TABLE 2. Levels of yeast cells in feces and gut following different routes of inoculation[a]

Group (no. of rats)	Mean CFU (10^5 CFU/g) ± SD								% of rats colonized		
	Feces			Colon	Cecum	SI-2	SI-1	Stomach	16 h p.i.	24 h p.i.	72 h p.i.
	8 h	16 h	24 h								
Group A (6)	313 ± 320	396 ± 260	341 ± 190	280 ± 170	130 ± 48	110 ± 65	48 ± 48	17 ± 11	NA[b]		NA
Group B1 (4)	0	0.146 ± 0.025	NA	0.075 ± 0.043	3.0 ± 4.2	1.7 ± 2.3	0.17 ± 0.11	0.12 ± 0.052	100		NA
Group B2 (2)	0	4.3 ± 3.3	1.6 ± 1.1	3.8 ± 3.6	6.2 ± 4.0	11 ± 5.5	0.17 ± 0.11	68 ± 88		100	NA
Group C1 (5)	0.024 ± 0.026	0.22 ± 0.14	NA	0.036 ± 0.074	0.019 ± 0.024	42 ± 64	0.051 ± 0.079	0.016 ± 0.037	60		NA
Group C2 (3)	0.015 ± 0.017	0.062 ± 0.07	0.022 ± 0.043	NA	NA	NA	NA	NA		33	33
Group D (8)	0.023 ± 0.011	0.95 ± 1.7	0	NA	NA	NA	NA	NA		0	25

[a] The sample consisted of feces or gut contents in all cases except the stomach contents, where the mucosa was scraped and included in the sample.
[b] NA, Data not available.

The yeast counts per gram of intestinal contents became more variable in control animals in the more caudal aspects of the gut. The greater mean value for colonic contents versus the cecal contents could be accounted for by the roughly twofold increase in dry matter in colonic contents as opposed the cecal contents. All dry weights of intestinal contents were about 25%, and the colon contents were about 40%. The greater number of CFU per gram of gut contents in the cecum versus the upper small intestine could be accounted for by the fact that most digestion and absorption occurs in the small intestine, with rodent chow being about 95% digestible energy and 90 to 95% capable of being metabolized (2). Intestinal contents in the upper small intestine of group A animals had a higher number of CFU per gram than in the stomach, probably due to the fact that the stomach had food contents that were less homogeneous due to the recently ingested food. Microbial levels compared well with those published by Kunstyr and co-workers (4, 5). The stomach volume in the forestomach-gastrectomized animals was smaller and had a lower pH than that of normal animals (5). Those two things could have accounted for the differences in yeast levels between the forestomach-gastrectomized and control animals.

Extra measures were taken to minimize the lag phase following inoculation of yeast cells, although one may have occurred following exposure to oxygen. Since intestinal transit time from duodenum to ileum takes 2 h (7), a lag phase of 1 h would not have taken the cells past the ileum.

Copraphagia did not appear to play a major role in yeast cell population changes. Only two of the eight rats became colonized following intrajejunal inoculation despite the fact that yeast cells were being shed for a period of 8 to 16 h. The oral route was the most likely source of inoculation in the two rats that became colonized, considering the fact that fecal yeast counts went to zero in both animals at 24 h p.i.

Retrograde stomach inoculation seemed most likely in intraduodenally inoculated animals, since colonization rates paralleled those of gastrically inoculated animals. Colonized intraduodenally inoculated animals also had high stomach yeast levels at 16 h p.i. Stomach colonization following intraduodenal inoculation occurred most frequently in smaller rats that probably had smaller lumens and more retrograde diffusion.

It could not be concluded that yeast cells did not divide in the proximal small intestine. Only half of the animals inoculated became colonized, and all of the animals that became colonized had high stomach yeast populations. At best, the total number of yeast cells in the duodenum probably would not have exceeded 40% of the total stomach population, making little contribution to yeast cell growth.

Dietary and environmental stress have been shown to significantly impact microbial populations (11) and the results of the laparotomy-inoculation-Bollman apparatus restraint, laparotomy-inoculation, and experimental milk diet regimens support this theory. The fact that only the Lewis rats lost their yeast population after milk was given suggests a difference between strains, a physical change in the gastric microenvironment, or a psychogenic effect influenced by previous adaptation to the Bollman apparatus. The lower fecal yeast levels for 2 months postlaparotomy and Bollman restraint suggests an irreversible change. Yeast levels in feces may be a sensitive indicator of rats under stress. The data underscore the absolutely essential need to precisely define environmental, previous psychological, and experimental conditions when documenting in vivo microbial populations.

Candida pintolopesii ferments glucose but not lactose. It was postulated that given the lower fecal yeast levels in the surviving Lewis rats, a 72-h restriction of yeast growth might allow it to wash out of the stomach. This appeared to be the case.

It was concluded that yeast cells distal to the duodenum were transient organisms which readily passed from the GI tract to the external environment. They were not able to colonize in these areas in conventional animals and probably would not grow considering the lack of increase in total yeast CFU following inoculation. Copraphagia was not an important contribution to stomach yeast cell populations, but environmental and dietary stress significantly impacted gut yeast levels. These studies demonstrate that yeast cells grow in the stomach environment, probably at rates comparable to in vitro growth rates, lending support to the idea that yeast doubling times are close to stomach half-emptying times.

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